

INVESTIGATING THE THROMBOPOIETIC SIGNIFICANCE OF PREPLATELET
MATURATION IN HEALTH AND ACQUIRED THROMBOCYTOPENIA

By

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Abstract

Circulating “preplatelets” (3-10 μ m in diameter) are platelet precursors that mature by transforming into barbell platelets and can undergo fission into two smaller platelets (2-3 μ m in diameter). In this study, we determine whether preplatelets are equivalent to immature platelets and whether they can transform into barbells and undergo fission into two mature platelets. By designing a new imaging cytometry methodology to accurately discriminate preplatelets and barbells in whole blood and in combination with microscopy, we have quantified and characterised these structures in humans and mice and demonstrated their analogy to immature platelets (displaying increased labelling with the immaturity markers thiazole orange and HLA I). Labelling with cytosolic dyes confirmed preplatelets exhibit the ability to transform into barbells and undergo fission. Accurate determination of immature platelets has the potential to non-invasively distinguish peripheral thrombocytopenia from dysfunctional platelet production. We demonstrate that preplatelet maturation events are significantly increased in response to enhanced peripheral destruction of platelets in human immune thrombocytopenia, acute thrombocytopenia in mice and absent following bone marrow ablation therefore, representing the rate of thrombopoiesis. Biotinylation in mice definitively proves that barbells are newly formed platelets. However, not all large platelets were immature, suggesting preplatelets are a subpopulation of large platelets.

200-word limit

Dedication

To my wonderful wife and boys

(The latter for helping me press buttons on my laptop)

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List of Abbreviations

A	Area
AA	Aplastic Anaemia
ACD	Acid Citrate Dextrose
ADAM17	ADAM Metallopeptidase Domain 17
ADAMTS13	A Disintegrin and Metalloproteinase - Thrombospondin Type 1 Motif 13
ADP	Adenosine Diphosphate
AF	Alexa Fluor
AMR	Ashwell Morell Receptor
APC	Allophycocyanin
APS	Anti-Platelet Serum
ATP	Adenosine Triphosphate
BAFF	B-cell Activating Factor
BCL	B-cell Lymphoma
BF	Brightfield
BH3	Homology 3
BHF	British Heart Foundation
BM	Bone Marrow
BMSU	Biomedical Services Unit
BSS	Bernard Soulier Syndrome
CCD	Charge-Couple Device
CCH	Centre of Clinical Haematology
Cdc42	Cell Division Cycle 42
CDK	Cyclic Dependent Kinase

CDKN1A	Cyclic-Dependent Kinase Inhibitor 1
CFC	Conventional Flow Cytometry
CH	Channel
CIE	Crossed Immunoelectrophoresis
Cif	Compensation Image File
CMP	Common Lymphoid Progenitor
COX1	Cyclo-Oxygenase
CPMS	Central Portfolio and Management
CRN	Clinical Research Network
CS&T	Cytometer Setup and Tracking
CSF	Colony Stimulating Factor
CTIR	Centre for Translational Inflammation Research
CXCR4	Chemokine Receptor-4
Daf	Data Analysis File
DC	Dendritic Cell
ddH ₂ O	Deionised Water
DMS	Demarcated Membrane System
DNA	Deoxyribonucleic Acid
DTS	Dense Tubular System
E4	Erode Plus 4 Pixels (Mask)
EB3	End-Binding Protein 3
EC	Endothelial Cell
ECM	Extracellular Matrix
EDTA	Ethylenediamine Tetra-Acetic Acid

EGFP	Enhanced Green Fluorescent Protein
Epo	Erythropoietin
EYFP	Enhanced Yellow Fluorescent Protein
F	Factor
F-actin	Actin Filament
F-Actin	Filamentous Actin
FcR γ -chain	Fc Receptor γ -chain
FGF-4	Fibroblast Growth Factor-4
FITC	Fluorescein Isothiocyanate
fL	Femtolitre
FLNA	Fliamin A
FMO	Fluorescence Minus One
FOG1	Friend of GATA1
FSC	Forward Scattered Light
GFP-EB3	Green Fluorescent Protein EB3
GlcNAc	N-acetylglucosamine
GlcNAc	N-acetylglucosamine
GM-CSF	Granulocyte and Macrophage CSF
GP	Glycoprotein
GT	Glanzmann Thrombasthenia
H	Height
HC	Healthy Control
HCV	Hepatitis C Virus
HESC	Human Embryonic Stem Cells

HIV	Human Immunodeficiency Virus
HLA I	Human Leukocyte Antigen I
HPSC	Human Pluripotent Stem Cells
HSC	Haematopoietic Stem Cells
IBR	Institute of Biological Research
IDEAS	Image Data Exploration Analysis Software
IFC	Imaging Flow Cytometry
IFN- γ	Interferon- γ
Ig	Immunoglobulin
IL-11	Interleukin 11
IL-1 α	Interleukin 1 α
IL-3	Interleukin 3
IL-6	Interleukin 6
IMS	Invaginated Membrane System
IP	Intraperitoneal
IPF	Immature Platelet Fraction
IRAS	Integrated Research Application System
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITP	Immune Thrombocytopenia
iTreg	Induced T-Regulatory Cell
IV	Intravascular
IVC	Individually Ventilated Cages
IVIG	Intravenous Immunoglobulin G
JAK1	Janus Kinase 1

JAK2	Janus Kinase 2
LAMP2	Lysosomal Associated Membrane Protein 2
LBD	Ligand Binding Domain
LEAM	Lomustine, Etoposide, Cytarabine and low dose Melphalan
LIBS	Ligand-Induced Binding Sites
LMPP	Lymphoid-Primed Multipotent Progenitor
LOX12	Lipoxygenase
LRR	Leucine Rich Repeat
LT-HSC	Long-Term Self-Renewing HSC
M	Morphology
Mab	Monoclonal Antibody
Maj-Ax-Int	Major Axis Intensity
MAPS	Microtubule Associated Proteins
MARKS	Myristoylated Alanine-Rich c Kinase Substrate
MDS	Myelodysplastic Syndrome
MEP	MK / Erythroid Progenitor
MFI	Median Fluorescence Intensity
MHC I	Major Histocompatibility I
Min-Ax-Int	Minor Axis Intensity
MK	Megakaryocyte
MLC2	Myosin Light Chain 2
MMP	Matrix Metalloproteinase
MPV	Mean Platelet Volume
mRNA	Messenger Ribonucleic Acid

MYH9-RD	MYH9-Related Disorders
Neu	Neuraminidase
NHS	(N-HydroxySuccinimido)
NHS REC	National Health Service Research and Ethics Committee
NIHR	National Institute for Health and Research
NO	Nitric Oxide
NQEH	New Queen Elizabeth Hospital
nTreg	Natural T-Regulatory Cell
OCS	Open Canalicular System
OXPHOS	Oxidative Phosphorylation
PAF	Platelet-Activating Factor
PBS	Phosphate Buffered Saline
PCT	Platelet Crit
PDW	Platelet Distribution Width
PE	Phosphatidylethanolamine
PGI ₂	Prostaglandin I ₂
PI(4, 5)P ₂	Phosphatidylinositol-4,5 Biphosphate
Pi3K	Phosphatidylinositol 3-Kinase
Pi3K-C2a	Phosphatidylinositol 4-Phosphate 3-Kinase C2a
Pi3P	Phosphatidylinositol 3-Phosphate
PKC	Protein Kinase C
PKA	Protein Kinase A
PLA	Platelet / Leukocyte Aggregate
PLC γ ₂	Phospholipase C γ ₂

PLT-F	Platelet Fluorescence
PLT-I	Platelet Impedance
PLT-O	Platelet Optical
PM	Plasma Membrane
PPP	Platelet Poor Plasma
PRP	Platelet Rich Plasma
PS	Phosphatidylserine
R	Region
RAMPS	Rabbit Anti-Mouse Platelet Serum
RBC	Red Blood Cells or Erythrocytes
RD	Relative Difference
retPLT	Reticulated Platelet – Abbott Diagnostics terminology.
RhoA	Ras Homolog Family Member A
Rif	Raw Image File
RMS	Root Mean Squared
RNA	Ribonucleic acid
ROCK	RhoA Kinase
RP	Reticulated Platelet
SCF	Stem Cell Factor
SDF-1	Stromal-Derived Factor-1
SFL	Side Scattered Fluorescence Intensity
SIM	Structured Illumination Microscopy
SLE	Systemic Lupus Erythematosus
SNPs	Single Nucleotide Polymorphisms

SSC	Side Scattered Light
ST-HSC	Short-Term self-renewing HSC
STn	Skeleton Thin
Sym2	Symmetry2
TDI	Time Delayed Integration
TF	Tissue Factor
TGF	Transforming Growth Factor
Th	T-helper
TO	Thiazole orange
TPO	Thrombopoietin
TRAP	Thrombin Receptor Activating Peptide
UOB	University Birmingham
UHB	University Hospitals Birmingham
ULVWF	Ultra Large VWF
UV	Ultraviolet
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	Very Late Antigen-4
VWF	Von Willebrand Factor
WBC	White Blood Cell
WT	Wild Type
Xkr8	Xk Related Protein 8

List of Publications

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Other:

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Chapter 1 - Introduction

1.1 Platelets

Platelets are anucleated, discoid-shaped blood cells approximately 2-3 μ m in diameter. They are one of the three main cellular constituents in blood and under normal physiological conditions play a critical role in haemostasis and thrombus formation, but in pathology mediate thrombosis or spontaneous bleeding when dysfunctional. Platelets patrol the bloodstream in a quiescent state and at sites of vascular damage, adhere, become activated, change shape, aggregate and form a thrombus to prevent significant blood loss. In addition to haemostasis, recent evidence suggests platelets also play a crucial role in inflammation and immunity [1-3].

As platelets lack a nucleus they rely on a rare (<0.1%), bone marrow (BM) derived progenitor cell, the megakaryocyte (MK) for their formation and release into the bloodstream [4]. Under normal physiological conditions the total platelet count is 150-450 x 10⁹/L in humans and 700-1000 x 10⁹/L in mice, with short lifespans of 7-10 days and 4-5 days respectively [5-7]. Abnormalities in platelet counts result in either thrombocytopenia (low counts) or thrombocytosis (high counts) resulting in increased risk of bleeding or thrombosis respectively.

1.1.1 Platelet Ultrastructure

Platelets are characterised by a highly invaginated plasma membrane (PM) surrounded by a glycocalyx, a complex cytoskeletal architecture consisting of spectrin, actin and microtubules, unique membrane receptors, an open canalicular system (OCS), dense tubular system (DTS), three secretory granules (α , δ and lysosomal) and respiring mitochondria [8, 9] (Figure 1.1).

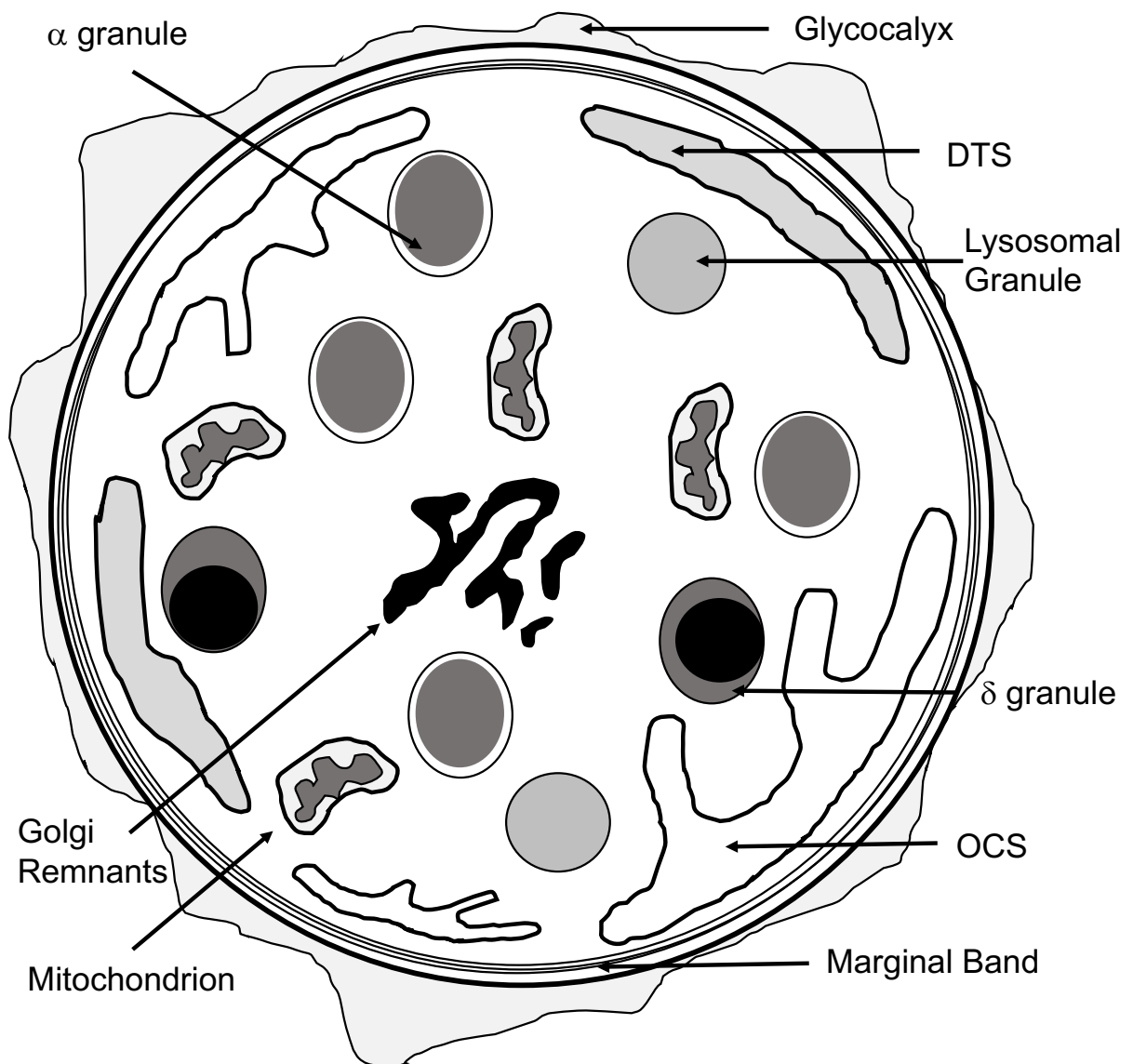


Figure 1.1: Platelet Ultrastructure Features Observed in the Cross Section of a Resting Discoid Platelet

OCS: open canalicular system; DTS: dense tubular system.

Compared to other blood cells, platelets have a thick outer membrane glycocalyx layer with open canalicular pores and a dense plethora of receptors distributed throughout [10]. Directly underneath the glycocalyx is the PM and like other cells is comprised of a phospholipid bilayer which has embedded glycolipids, glycoproteins and cholesterol.

The PM is anchored by an actin/spectrin cytoskeletal matrix [11]. Under resting conditions, phospholipids are asymmetrically positioned in the inner leaflet of the PM. Aminophospholipids such as phosphatidylserine (PS) and phosphatidylethanolamine (PE) are predominantly expressed in the inner leaflet. The location of the aminophospholipids ensures the platelet remains in an inactive state. This membrane asymmetry is maintained by flippases and floppases. Once activated PS is translocated to the PM outer leaflet by scramblase [12, 13]. PS exposure plays a critical role in facilitating the assembly of coagulation factors and therefore thrombin generation to support haemostasis [14].

The OCS was first described in rats by Benhke in 1967 [15]. This interconnected tubular system is a continuation of the plasma membrane consisting of the same lipid and glycoprotein composition. The OCS has three possible functions: a potential route for granule release, a reservoir pool of membrane to facilitate platelet morphogenesis and spreading during activation and/or a storage site for plasma membrane glycoproteins such as GPIIb α [16, 17].

Platelets also contain a DTS however, this is not connected to the plasma membrane [18]. The DTS is derived from the endoplasmic reticulum of MKs. The long, thin tubules of the DTS regulate platelet activation by either sequestering or releasing calcium (Ca^{2+}) from its stores facilitating the regulation of platelet activation [19, 20].

Platelet granules are situated in the organelle zone. Three main types of granules have been described: α , δ and lysosomal granules [8]. The α -granules are the most abundant platelet granule (50-80 per platelet and these granules are 200-500nm in diameter) and contain both membrane bound and soluble proteins including factor V, von Willebrand Factor (VWF) and p-selectin (CD62p) which play critical roles in

haemostasis [21-24]. For example, upon activation, CD62p is translocated to the surface and platelets situated at the core of a thrombus become irreversibly positive [25]. In addition to haemostasis, α -granular proteins also play a role in platelet/leukocyte interactions, wound healing and angiogenesis [26, 27].

Dense granules are less abundant (3-8 per platelet) and smaller in size (250nm in diameter) compared to α -granules [23]. Dense granules store adenosine triphosphate (ATP), adenosine diphosphate (ADP), serotonin, polyphosphate, Ca^{2+} and magnesium which all support haemostasis [28].

The third platelet granules are termed lysosomal granules [29]. A single platelet contains 0-2 lysosomal granules which store acid hydrolases, cathepsins, lysosomal associated membrane protein (LAMP)-1 & 2 and CD63. As platelet lysosomes contain proteolytic enzymes it is postulated they play a role in thrombus remodelling [30]. Recent evidence also suggest they are involved in platelet turnover [31].

Platelets contain fully functional mitochondria. Unlike other nucleated cells which contain hundreds of mitochondria, platelets only contain 5-8 [32]. However, like other nucleated cells these mitochondria contain DNA, are encased in a membrane bi-layer and undergo fusion or fission [33]. Furthermore, they have been shown to support metabolism, activation, ATP production required for cellular processes and controlling lifespan (the latter described in "Platelet Lifespan and Clearance") [34, 35]. To meet resting energy demands, platelet mitochondria perform a high rate of ATP turnover where 60% is thought to originate from glycolysis and 40% from oxidative phosphorylation (OXPHOS) [36].

1.1.2 Platelet Receptors

Platelets interact with their external environment via a multitude of membrane receptors. The agonistic and inhibitory potential of these receptors is essential for regulating platelet responses to vessel wall damage as well as platelet/platelet interactions. The platelet PM houses a dense population of receptors. These receptors are primarily involved in adhesion, activation, immunology and inflammation. Examples of platelet receptors and their main functions can be found in Table 1.1.

Table 1.1: Platelet Receptors and Main Functions

FAMILY	RECEPTOR	FUNCTION
Integrin $\beta 1$	$\alpha 2\beta 1$	Adhesion
Integrin $\beta 1$	$\alpha 5\beta 1$	Adhesion
Integrin $\beta 1$	$\alpha 6\beta 1$	Adhesion
Integrin $\beta 3$	$\alpha v\beta 3$	Adhesion
Integrin $\beta 3$	$\alpha_{IIb}\beta 3$	Aggregation
Leucine-Rich Repeat	GP1b-IX-V Complex	Adhesion
Leucine-Rich Repeat	Toll-like Receptors	Inflammation
GPCR	PAR1, 4	Activation
GPCR	P2Y1, P2Y12	Degranulation
GPCR	TX	Activation
GPCR	PGI ₂	Inhibition
GPCR	PAFr	Activation
GPCR	Chemokine Receptors	Trafficking
Ig Superfamily	GPVI	Activation
Ig Superfamily	Fc γ RIIa (CD32)	Clearance
Ig Superfamily	Fc ϵ RI (CD23)	Infection
Ig Superfamily	G6B	Inhibition
Ig Superfamily	CD148	Inhibition

Ig Superfamily	PECAM-1	Inhibition
Ig Superfamily	TLT-1	Activation
Ig Superfamily	CD47	Activation
C-Type Lectin Receptor	P-Selectin (CD62p)	Leukocyte Binding
C-Type Lectin Receptor	CLEC-2	Activation
Tetraspanins	CD9 (Tspan29)	Inhibition
Tetraspanins	CD63 Tspan30)	Activation
Tyrosine Kinase	c-Mpl	Sequesters TPO
Other	CD36	Activation
Other	LAMP1 & 2	Thrombus Structure

GPCR: G Protein-Coupled Receptor; **Ig:** Immunoglobulin; **GP:** Glycoprotein; **PAR:** Protease Activated Receptor; **TX:** Thromboxane; **PGI:** Prostacyclin; **PAFr:** Platelet-Activating Factor receptor; **PECAM-1:** Platelet Endothelial Cell Adhesion Molecule-1; **TLT-1:** Trem-Like Transcript-1.

Due to their important role in haemostasis, the three of the most studied glycoprotein receptors on the platelet surface are $\alpha_{IIb}\beta_3$, GPIb-IX-V and GPVI and will be described in more detail below and representative diagrams can be viewed in Figure 1.2.

The integrin $\alpha_{IIb}\beta_3$ is the most abundantly expressed platelet membrane receptor (80,000 copies per platelet) and accounts for 3% of total platelet protein mass and 17% of total membrane mass [37]. In platelets, this integrin operates as a receptor which bridges platelets together through binding $\alpha_{IIb}\beta_3$ -bound ligands with adjacent platelets and subsequently promoting aggregation. As $\alpha_{IIb}\beta_3$ is indispensable in aggregation, in cardiovascular disease this receptor has become a key target for antithrombotic therapy [38].

Like all integrins, $\alpha_{IIb}\beta_3$ is a noncovalent heterodimeric receptor and consists of two subunits α_{IIb} and β_3 (Figure 1.2b), otherwise known as CD41 and CD61 respectively. The genes which encode $\alpha_{IIb}\beta_3$ subunits are ITGA2B and ITGB3 respectively. Various

single point mutations of either or both genes give rise to haematological disorders. The most studied of these is Glanzmann Thrombasthenia (GT) [39]. The severe bleeding symptoms related to GT outline the indispensable role $\alpha_{IIb}\beta_3$ plays in haemostasis.

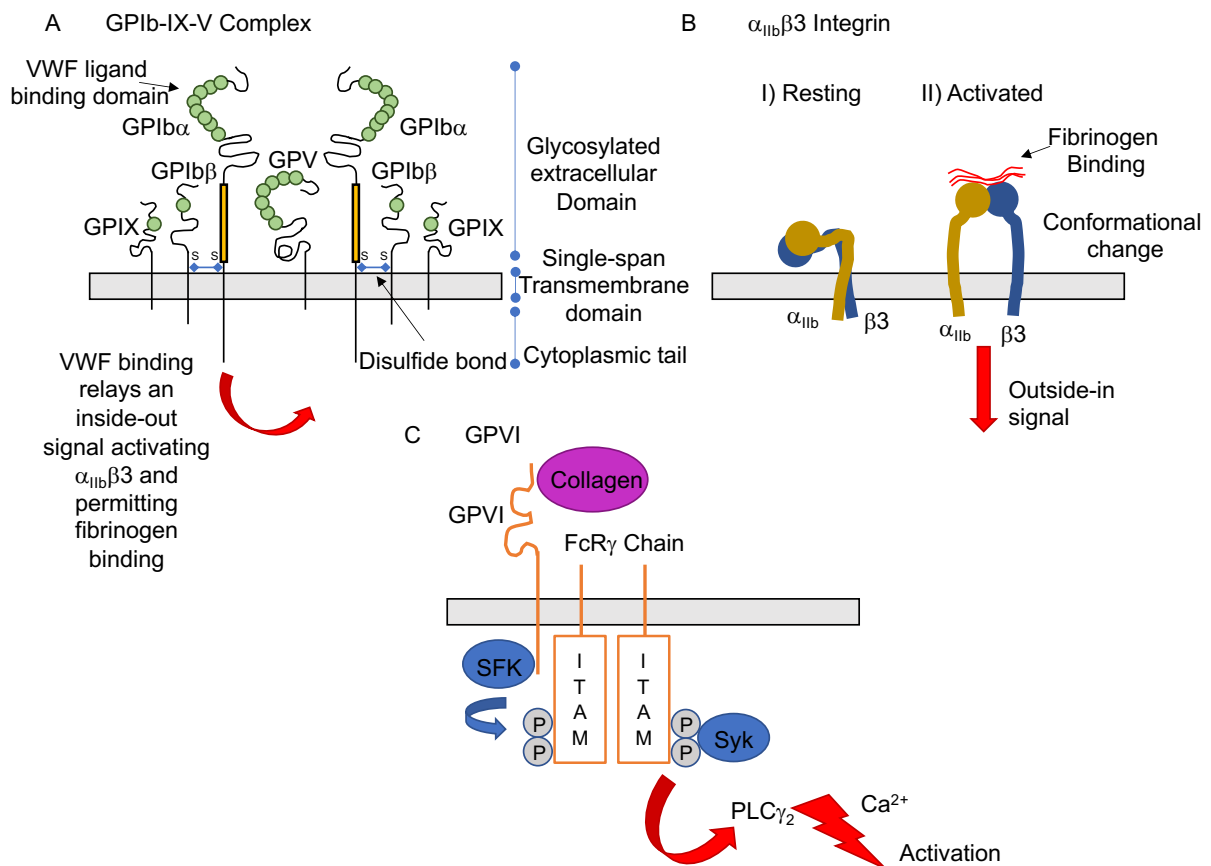


Figure 1.2: Platelet Receptor Schematics

(A) GPIb-IX-V complex consists of 4 subunits: GPIb α , GPIb β , GPIX and GPV where GPIb α is linked to GPIb β via a disulfide bridge. GPIb α contains the ligand binding domain where its major ligand is von Willebrand factor (VWF). During the initiation of haemostasis, VWF bound to the damaged vessel wall binds to GPIb α and triggers inside-out signalling, activating the $\alpha_{IIb}\beta_3$ integrin and subsequently promoting strong platelet adhesion. (B) When resting, ligand binding to $\alpha_{IIb}\beta_3$ is inhibited. In response to inside-out signalling, $\alpha_{IIb}\beta_3$ changes conformation and permits ligand (such as fibrinogen) binding. Ligand binding relays an outside-in signal to trigger platelet aggregation and plug formation. (C) As platelets strongly adhere to the vessel wall this permits GPIIb/IIIa/collagen signalling. GPIIb/IIIa is an immunoglobulin (Ig) superfamily receptor associated with a constitutively expressed Fc receptor γ -chain (FcR γ chain) which contains an immunoreceptor tyrosine-based activation motif (ITAM). Upon activation, FcR γ chain is phosphorylated by src family kinase (SFK) on the tyrosine residue on ITAM, which recruits and activates spleen tyrosine kinase (Syk) and ultimately triggers a cascade of signalling events resulting in phospholipase C γ_2 (PLC γ_2), Ca $^{2+}$ mobilisation and strong platelet activation.

$\alpha_{IIb}\beta_3$ recognises a simple peptide sequence Arg-Gly-Asp (RGD) on multiple ligands: fibrinogen, fibronectin, vitronectin, thrombospondin-1 and VWF [40]. Fibrinogen is the major ligand and this association not only facilitates platelet-platelet interactions it also relays outside-in signals which play a key role in clot stabilisation and retraction [41]. Ligand binding can also cause conformational change, creating ligand-induced binding sites (LIBS) which are thought to be a mechanism behind immune thrombocytopenia targeting $\alpha_{IIb}\beta_3$ [42].

The second most abundant platelet receptor is the GPIb-IX-V complex with approximately 25,000 copies per platelet [43, 44]. GPIb-IX-V is responsible for tethering platelets to the injured vessel wall under shear flow, causing platelet activation and ultimately thrombus formation. GPIb-IX-V is exclusively MK lineage-specific. This leucine-rich repeat (LRR) glycoprotein (GP) complex is comprised of 4 subunits: GPIb α (heavy chain), GPIb β (light chain), GPIX and GPV [45] (Figure 1.2a). Individual subunits are encoded by separate genes and are type I transmembrane proteins which contain a glycosylated extracellular domain, a single-span transmembrane domain and a cytoplasmic tail. GPIb α is disulfide-linked to GPIb β (together known as GPIB) and are non-covalently associated with GPIX and GPV [46, 47]. However, expression of GPIB is dependent on GPIX and not GPV.

GPIb α is the major ligand-binding subunit. At sites of vascular damage GPIb α adheres to its major ligand, VWF on the subendothelial matrix through rolling and transient interactions. VWF is an ultra large multimeric glycoprotein (up to > 20,000 KDa) composed of multiple copies of a single subunit consisting of 2813 amino acids [48, 49]. VWF is synthesised by MKs and endothelial cells (ECs) and can either be stored as ULVWF (ultra large VWF) in platelet α -granules or EC Weibel Palade bodies or

secreted constitutively into the blood and sub-endothelium [50, 51]. The primary source of plasma VWF is from EC, although activation of both platelets and EC pools results in the transient release of ULVWF to further promote platelet/vessel wall interactions [52]. The greater the size or multimer number of VWF the greater the binding avidity to the vessel wall and to platelets and GPIb α [53].

VWF consists of A1 and A3 domains which contain the GPIb α and vessel wall collagen type I/III binding sites respectively [54]. Shear force is also essential for GPIb/VWF tethering. Under low flow rates VWF exists as a loosely coiled “ball of wool” conformation. However, when VWF binds to collagen under high shear, VWF unravels into a linear conformation exposing multiple binding sites in each subunit including the A1 and A2 domains [55]. The A2 region also contains the cleavage site for ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif) [56]. Proteolytic cleavage by ADAM13 at the A2 domain of ULVWF releases smaller lengths of VWF to form the classical multimeric pattern in normal plasma.

Binding of GPIb-IX-V to VWF can also initiate GPIb α outside-in signalling, activating $\alpha_{IIb}\beta_3$ and promoting aggregation [57]. The clinical importance of GPIb-IX-V/VWF association can be best described in patients with a rare platelet disorder Bernard Soulier Syndrome (BSS). This inherited macrothrombocytopaenia is characterised by a low platelet count and giant platelets with a severe bleeding diathesis which indicates the importance of GPIb-IX-V not only in haemostasis but for normal platelet production and regulation of platelet size.

Finally, GPVI is a major collagen receptor and is the key driver of mediating integrin firm adhesion to the vessel wall through inside-out signalling. Like GPIb-IX-V, GPVI is exclusively expressed in platelets and megakaryocytes however, the copy number is

much less (3000-4000 copies per platelet) [58]. GPVI is an immunoglobulin (Ig) superfamily receptor associated with a constitutively expressed Fc receptor γ -chain (FcR γ -chain) which contains an immunoreceptor tyrosine-based activation motif (ITAM) [59]. For GPVI signalling to occur, the FcR γ -chain is phosphorylated by src family kinase on the tyrosine residue on ITAM (Figure 1.2c). This triggers a cascade of signalling events which result in modulation of phospholipase C γ_2 (PLC γ_2) and ultimately Ca² mobilisation and protein kinase C (PKC) activation [60, 61].

GPVI is now recognised as a novel anti-platelet target. As the copy number per platelet is low, therapeutically targeting GPVI inhibits collagen induced platelet activation without causing a significant increase in bleeding [62, 63]. There are currently several humanised drugs either undergoing clinical trials or have been used within a clinical setting [64].

1.1.3 Platelets in Haemostasis

In the absence of vascular damage, the platelet is maintained in a resting state by a multitude of antithrombotic constituents in the blood. For example, EC inhibit platelet activation by secreting prostaglandin I₂ (PGI₂) and nitric oxide (NO) [65-67]. However, in the event of vascular breach, the endothelial vessel wall releases vasoconstrictors renin, endothelin and platelet activating factor (PAF) [68, 69]. This, narrows the lumen space, slows down bleeding and ultimately facilitates increased platelet/EC interactions and activation.

In the event of vascular damage, platelets are exposed to a diversity of adhesive macromolecules such as collagens, VWF, fibronectin, laminin and thrombospondin. These ligands bind to their respective platelet-bound receptors and permit platelet

tethering to the vessel wall [70]. Once the velocity of a platelet is slowed down, they can firmly adhere to the subendothelium, become activated and commence primary haemostasis.

The mechanism which platelets tether to the damaged vessel is dependent on shear forces [71-73]. Such forces negatively correlate with blood vessel diameter [74]. In veins or large arteries, the shear rate is low ($<600\text{-}900\text{s}^{-1}$), and platelets can adhere directly to collagen, fibrinogen, fibronectin and laminin through $\alpha_{IIb}\beta_3$ and other receptors [75]. However, in small arteries and micro-capillaries where shear rate is high ($>6000\text{s}^{-1}$), platelets rely on GPIb-IX/VWF tethering to slow the platelets down and permit activated $\alpha_{IIb}\beta_3$ to also interact with VWF and the vessel wall.

When exposed to high shear stress, VWF adheres to collagen, becomes immobilised and is extended to expose multiple A1 domain binding sites for GPIb-IX binding [76]. Platelets are thus slowed down under high velocity and roll on the vessel wall via rapid on and off binding [77, 78], generating a weak activation signal to induce inside-out signalling and trigger firm adhesion to extracellular matrix (ECM) proteins such as VWF and fibrinogen through $\alpha_{IIb}\beta_3$ and to collagen by $\alpha_2\beta_1$ [79].

GPVI also plays a central role in collagen binding, activating multiple adhesive integrins including $\alpha_2\beta_1$ [80, 81]. GPVI binds to specific glycine-proline-hydroxyproline (GPO) sites on collagen [82]. In response to GPVI activation, Ca^{2+} mobilisation is induced, platelets release α and δ granule contents and trigger a positive feedback signal to enhance platelet activation through $\alpha_2\beta_1$ and $\alpha_{IIb}\beta_3$ signalling [83].

Following integrin-mediated activation, platelets bind to the site of vascular damage and spread, forming a stable monolayer. These activated platelets generate secondary signals and thus release α and δ granules to activate and recruit passing platelets.

These follower platelets tether and bind to adhered platelets via GPIb-IX and $\alpha_{IIb}\beta_3$ -bound VWF. However, as activation of recruited platelets cannot be induced through GPVI/collagen signalling, they become activated through exposure to platelet releasates containing ADP/ATP [84]. Secondary signals (e.g. Thromboxane) are generated through oxygenases COX1, LOX12 and granule mediators [85] to generate a positive feedback loop to maintain recruitment and enhance primary activation signals.

For clot stabilisation and vessel repair to occur, primary haemostatic mechanisms both interact with and promote secondary haemostasis. This process depends on the coagulation cascade which involves a series of multiple zymogen clotting factors [86, 87]. The cascade is composed of the intrinsic and extrinsic pathways. Damage to the vessel wall exposes tissue factor (TF) which in turn initiates the extrinsic or TF pathway [88, 89]. In contrast, the intrinsic or contact pathway is triggered by Factor (f)XII in contact with charged surfaces. Both signalling cascades converge into forming the tenase (TF/fVIIa extrinsic and fIXa/fVIIIa intrinsic) and prothrombinase (fIXa/fVa) complexes which convert prothrombin to thrombin. In turn, thrombin cleaves fibrinogen to fibrin monomers, which the fibrin stabilisation factor (fXIII) converts into insoluble fibrin polymer to stabilise the platelet clot.

1.1.4 Platelet Cytoskeleton

For circulating platelets to maintain a discoid shape under varied flow conditions, they require a highly engineered cytoskeleton. Platelets have three major cytoskeletal components: spectrin, actin and a tight peripheral coil of microtubules [11] (Figure 1.3).

On average, resting platelets contain 2 million copies of actin, 2000 spectrin fibres and microtubules composed of a total of $2.8\text{-}3.2 \times 10^5$ tubulin subunits.

1.1.4.1 Spectrin

Spectrin is critical for forming a robust platelet membrane skeleton to maintain resting discoid shape. Spectrin, adducin and actin filaments make up this structure and are situated directly underneath the plasma membrane [90] (Figure 1.3aI & b). The main spectrin subunits are α II and β II. These fibres, inter-link and bind with the ends of long actin-filaments and cross-link with filamin A (FLNA) and actin binding proteins (ABP), which create hetero-tetramers (triangle-like pores) [91]. Adducin binds to and caps actin barbed ends to facilitate its association. Formation of such triangular pores stabilises the spectrin-actin cytoskeleton and acts as a shell to secure the actin core.

1.1.4.2 Actin

Human platelets consist of three subtypes of actin which are expressed by 6 genes (3 α -, 1 β - and 2 γ -actin genes) [92-94]. Filamentous actin or actin filament (F-actin) consist of multiple G-actin monomers which are constructed in a head and tail manner (Figure 1.3aII). F-actin has plus, and minus ends otherwise known as barbed and pointed ends respectively. These regions differ in their affinity for ATP-bound G-actin and act as sites for assembly and growth or disassembly.

The arrangement of actin in resting platelets consists of a central hub with radial spindles which are associated with myosin. In the absence of haemostatic stimuli, only approximately forty percent of ATP-bound G-actin monomers are assembled into F-actin [95, 96]. Most of the remaining non-polymerised G-actin is situated in the platelet

cytoplasm and is bound to $\beta 4$ -thymosin complexes to prevent their attachment to the myosin (subfragment 1) associated pointed (minus) ends of actin filaments [97-99]. Furthermore, >98% barbed ends are capped by capZ and adducin proteins to prevent monomer assembly [100]. In contrast, most pointed ends are capped by Arp2/3 [101] (Figure 1.3b). As Arp2/3 is capable of nucleating new filaments from the lateral region of existing filaments it is hypothesised this protein is essential for resting platelet morphology [102].

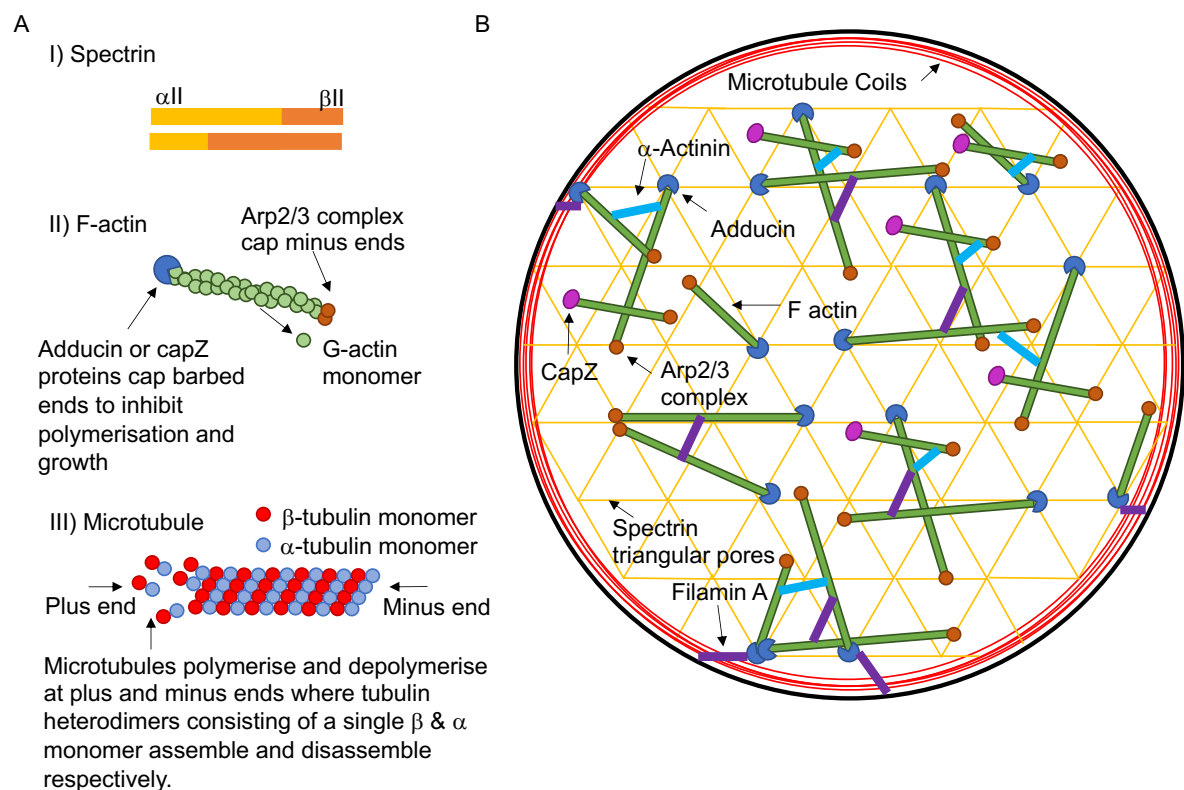


Figure 1. 3: Resting Platelet Cytoskeleton

(A) The platelet cytoskeleton consists of I) spectrin (consisting of α II & β II subunits), II) actin and III) microtubules. (A II) Multiple ATP-bound G-actin monomers bind to form F-actin. Under resting conditions, F-actin contains a barbed (capped by either adducin or capZ) and pointed end (capped by Arp2/3 complexes) where growth occurs at the barbed end. A III) Microtubules are polymers of tubulin heterodimers (consisting of one α and β monomer). Heterodimers assemble and disassemble at the plus and minus ends to form a tubular protofilament. Spectrin/actin association and microtubule coiling at the cortex is essential for maintaining resting platelet morphology. (B) Spectrin inter-links and binds with F-actin via adducin, filamin A and α -actinin forming a robust structure consisting of hetero-tetramer triangular pores. In contrast, microtubule polymer coils (8-12) are located directly under the cell membrane. This structure is termed the marginal band and is essential for maintaining platelet discoid shape.

As platelets express a high degree of crosslinker proteins such as FLN and α -actinin it is proposed the actin network of resting platelets is crosslinked at various locations to form a rigid complex [103-106]. Although platelets express both FLNA and B, the former is much more abundant by a factor of 10 [107-110]. FLN is a scaffold protein and consists of an N- and C-terminus where the N-terminus associates with actin and the C-terminus recruits multiple binding partners to the plasma membrane. These binding partners include small Rho GTPases RhoA, Rac, RalA and Cdc42 [111, 112]. Actin associates with the plasma membrane through FLN linking actin filaments to GPIB/IX subunits [110]. This interaction between FLN and GPIB/IX complex, is essential for structural organisation of resting platelets and morphogenesis when activated. Previous findings suggest >90% of FLN is associated with GPIb/IX [113]. This association is essential for platelet formation, release and size [114, 115]. Actin also associates with $\alpha_{IIb}\beta_3$ which subsequently induces actin polymerisation and cytoskeletal reorganisation [41].

1.1.4.3 Microtubules

Microtubules are long polymers which consist of globular α -tubulin and β -tubulin polypeptide heterodimers which polymerise at the head and tail region to form protofilaments. Assembled protofilaments form a tubular structure (~25nm in diameter) with α -tubulin and β -tubulin exposed at either the minus or plus ends respectively (Figure 1.3aIII). Microtubule growth occurs at the plus end and the rate of assembly relates to the rate of polymerisation and depolymerisation. In a resting platelet >50% of tubulin is polymerised [116]. Microtubule assembly is also affected by catastrophic,

rescue events and latent periods which are controlled by microtubule associated proteins. Platelets have also been shown to express γ -tubulin [117].

In resting platelets, tubulin forms a tightly coiled microtubule polymer bundle at the periphery, 8 -12 coils thick [118, 119]. This circumferential structure is termed the marginal band and is located below the plasma membrane (Figure 1.3b). Unlike nucleated cells, platelets lack a microtubule organisation centre thus, microtubules are thought to be nucleated by γ -tubulin seeds situated in the marginal band [117].

The primary function of the marginal band is to maintain resting platelet discoid shape. Human studies have shown when disrupting microtubule signalling with nocodazole, colchicine or vincristine as well as inflicting disassembly of microtubules through exposure to 4°C platelets lose their discoid shape [20, 120]. Furthermore, mice which lack specific expression of tubulin isoforms also have spheroid platelets (described below) [121].

Due to limitations in static imaging, for many years, scientists believed the marginal band consisted of a single inactivated microtubule (100 μ m in length), coiled 8-12 times [118, 119]. However, Patel-Hett and co-workers (2008) discovered when labelling microtubules with EB1, this end-binding protein localised at approximately 9 plus ends on microtubules situated within the marginal band of resting platelets [117]. Using the green fluorescent protein EB3 (GFP-EB3), time-lapse fluorescent microscopy revealed, microtubules in the marginal band continuously assemble in a bidirectional manner. Furthermore, this highly dynamic structure consisted of a single mature acetylated microtubule and multiple new tyrosinated microtubules with varying degrees of polarity. These researchers predict, half of the marginal band tubulin content is in the polymer form making the other half a soluble pool. Due to dynamic instability, where

microtubules assemble and disassemble at the plus end, the soluble store is maintained. During activation, it is thought the soluble pool is rapidly depleted. This suggests, the dynamic nature of marginal band primes the resting platelet cytoskeleton making it prepared for rapid morphogenesis when exposed to activation cues.

Megakaryocytes and platelets have been proven to express four β -tubulin isoforms β 1, β 2, β 4 and β 5 [121]. Little is known regarding α -tubulin with α 4 being the only isoform investigated [122]. By knocking down the tubulin gene *TUBB1*, Schwer and colleagues (2001) discovered that *TUBB1* deficient mice lacked the β 1-tubulin isoform in MK and platelet lineages only [121]. In addition to linear restriction, this group showed β 1-tubulin accounts for ~90% of the total microtubule content in platelets and in the absence of this isoform mice developed thrombocytopenia due to a dysfunction in the final stages of MK maturation (proplatelet formation). In contrast, β 1-tubulin deficient platelets from this mouse displayed a defect in microtubule constructs, including frequent kinks and breaks, and marginal bands consisting of fewer coils (2-3) causing spherocytosis. In the absence of β 1, expression of isoforms β 2 and β 5 increased. However, as these failed to rescue the phenotype it suggested, β 1 is the dominant platelet β -tubulin isoform. In addition to defects in resting morphology, upon thrombin exposure, β 1-tubulin deficient platelets failed to reorganise their cytoskeleton and spread on surface substrates.

Phenotypical analysis of the β 1-tubulin knockout mouse model confirms β 1-tubulin is essential for maintaining the platelet discoid shape but not overall size, granularity and function [123]. Interestingly, from the β 1-tubulin $-/-$ mouse colony, heterozygous $(+/-)$ mice have no defect in platelet morphology and ability to spread, suggesting that

greater than 50% of the basal tubulin level is required to maintain marginal band isotubulin stability and thus, discoid cell shape [121, 123].

It is thought the dominant α -tubulin isoform in platelets is α 4A [124]. Like β 1-tubulin, α 4A-tubulin is upregulated during thrombopoiesis. Single nucleotide polymorphisms (SNPs) in the α 4A gene results in macrothrombocytopenia. Such large platelets consist of deformities of the marginal band. Defects were also observed during MK maturation events suggesting normal expression of this α -tubulin isoform is critical for normal platelet formation.

Microtubules also play a key role in proplatelet formation and platelet release. This is discussed in detail in section 1.2.2.

1.1.4.4 The Cytoskeleton in Response to Activation

Upon activation, platelets transform from discs to spheres and form filopodia structures which extend over the subendothelium, become adhered, flattened and subsequent gaps are filled by the extruded OCS which forms lamellipodia [125, 126]. The remaining internal components are compressed at the central region of the platelet through cytoskeletal contraction forces thus, taking on a fried egg-like morphology. Upon contact, two spread platelets form tight junctions which prevent significant blood loss [127]. Extended filopodia also contribute to the total surface area for recruited platelets to bind, promoting stable thrombus formation.

Platelet shape change and subsequent spreading requires rapid cytoskeletal turnover and reorganisation. For this to occur, spectrin becomes disassociated from actin and the PM and subsequently centralised [128]. Simultaneously, the marginal band coils and depolymerise to form a smaller proximal ring [129]. Resting platelets have ~35%

of F-actin, which is almost doubled in response to platelet activation [130, 131]. Increases in cytosolic Ca^{2+} , activates gelsolin which cleaves F-actin to increase the number of barbed ends [132]. These ends are uncapped through Rho GTPase-mediated $\text{PI}(4, 5)\text{P}_2$ activation [133]. $\beta 4$ -thymosin and profilin carry actin monomers to free barbed ends to promote elongation [134]. Temporal regulation of actin polymerisation is achieved through the capping of new filament and depolymerisation of old filaments to construct new.

During platelet activation, myosin is rapidly activated and becomes associated with actin. The protein structure of myosin (or Myosin IIA) consists of a globular head region comprised of heavy chain and essential and regulatory light chain molecules, a neck and coiled rod region and finally a phosphorylated non-helical tailpiece [135]. Myosin is critical for maintaining cell morphology, cell adhesion and cytokinesis [136]. The myosin/actin association has been shown to form stress fibres in platelets to generate contractile forces [137]. Such forces are essential for spreading and aggregate stability and have been implemented in clot retraction and regulation of thrombus size.

1.2 Platelet Production

Platelets derive from MK which mainly reside in the BM. There are multiple theories regarding how MK progenitor cells differentiate from haematopoietic stem cells (HSC): 1) classical model, 2) MK-biased and 3) lymphoid-primed [138, 139] (See diagram in Figure 1.4a - c).

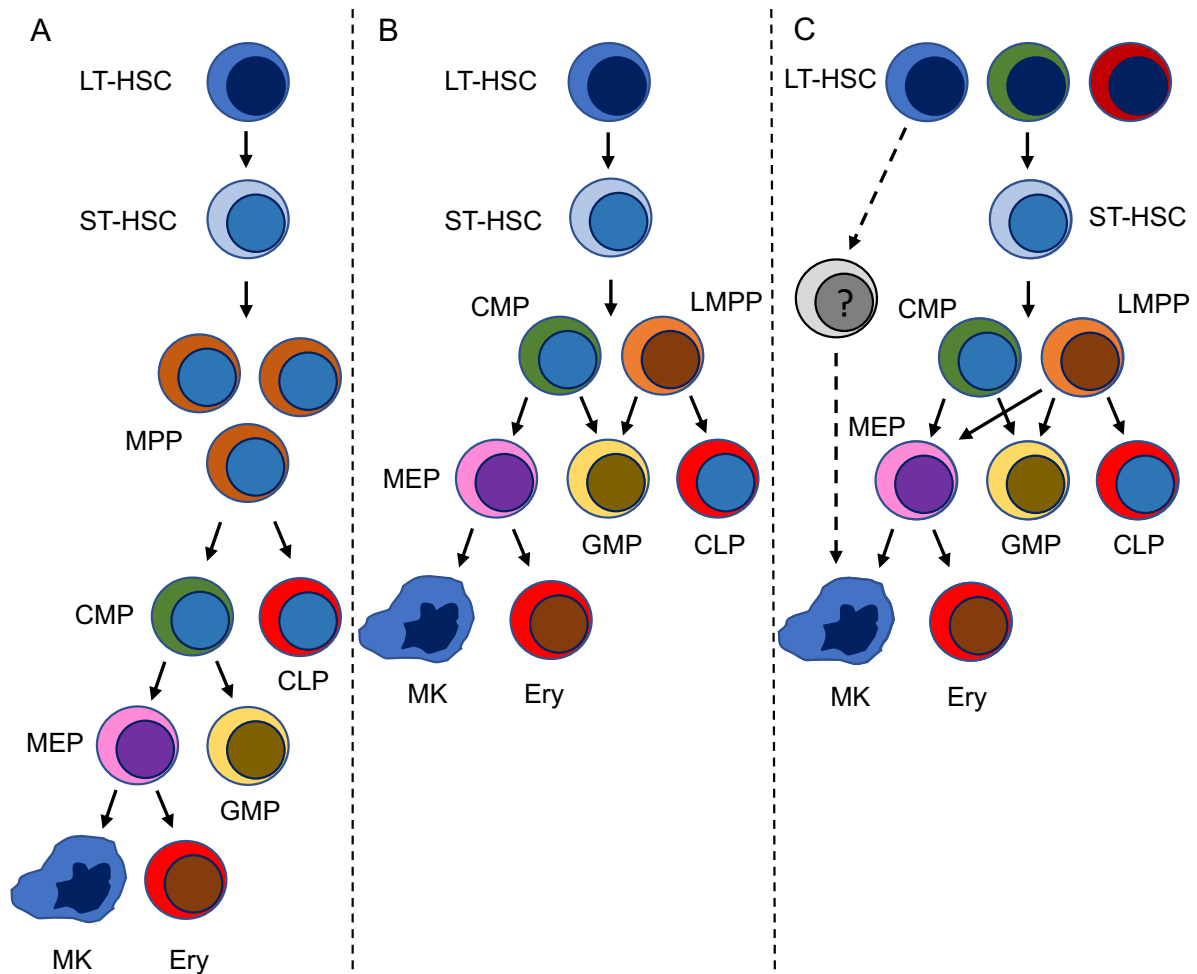


Figure 1.4: Models of Haematopoiesis

Figure adapted from Platelets 4th Edition (2019). (A) the classical model of haematopoiesis assumes there is complete segregation between lymphoid and myeloid lineages following commitment by multipotent progenitor cells (MPP). (B) The lymphoid-primed multipotent progenitor (LMPP) model sees an early loss of megakaryocyte (MK) commitment occurring at the short-term haematopoietic stem cell (ST-HSC) phase. LMPP give rise to lymphocytes (CLP) and granulocyte-macrophage (GMP). (C) predicts long-term- (LT-)HSC are heterogeneous in their lineage potential. From this it is hypothesised a MK-biased pathway occurs where VWF positive HSC bypass the classical intermediate commitment stages. CMP: common myeloid progenitor; MEP: megakaryocyte-erythroid progenitor; Ery: erythrocyte.

The classical model assumes there is strict segregation between lymphoid and myeloid lineages. In this model, long-term self-renewing HSC (LT-HSC) lose their ability to self-renew, become short-term self-renewing HSC (ST-HSC) and eventually multipotent progenitors where myeloid lineage commitment takes place through the common lymphoid progenitor (CMP) [140]. In turn, CMP give rise to bipotent MK/erythroid

progenitors (MEP) which give rise to MK progenitors. In contrast, the MK-biased model predicts the LT-HSC population is heterogeneous where VWF positive HSC bypass intermediate commitment stages and directly give rise to MK progenitors [141]. Finally, the lymphoid primed model sees an early loss of MK potential where ST-HSCs differentiate directly into lymphoid-primed multipotent progenitors (LMPP) which favour T, B and granulocyte lineages [142, 143]. These new emerging models of haematopoiesis have been shown to differ depending on the degree of platelet turnover [144, 145].

1.2.1 Megakaryopoiesis

During the early stages of megakaryopoiesis, MK switch from mitosis to endomitosis to increase total cell nuclear content, mass and size [146]. During this stage of maturation, MK construct a complex of cisternae and tubules distributed throughout the cytoplasm called the invaginated membrane system (IMS), formerly known as the demarcated membrane system (DMS; discussed below). Once fully mature, MK enter the final stages of maturation, migrate to the BM sinusoid micro-capillaries, form proplatelets which penetrate the vessel wall and release platelets into the bloodstream. Endomitosis occurs because of a failure in the late phase of cytokinesis, associated with an incomplete formation of the cleavage furrow [147]. As a result, MK increase ploidy and form a polylobulated nucleus. The degree of ploidy (4-64N) depends on the number of successive endomitotic cycles [148]. This increase in nuclear content provides the MK with enough mRNA and protein to produce up to ~2000 platelets per cell.

Following endomitosis, large, polyploid MK synthesise extensive amounts of protein and lipid which is used to construct the IMS [149]. This highly invaginated structure consists of cisternae and tubules and is protected and anchored by spectrin/actin tetramers [91]. The IMS functions as a reservoir of membrane for MK to produce multiple cytoplasmic extensions called proplatelets [150].

1.2.2 Proplatelet Formation

In 1976 Becker and DeBruyn suggested that platelets arise from elaborate MK-derived cytoplasmic extensions or proplatelets within the sinusoid vasculature [151]. This was further developed into a working model by Radley *et al* where it was stipulated platelets derive from demarcated platelet compartments within the DMS (the “territory model”) [152, 153]. However, multiple sections of MK cytoplasm, microscopic analysis confirmed the absence of platelet marginal bands situated within the so-called DMS suggesting platelets are synthesised de novo during proplatelet formation establishing the IMS “flow model” of thrombopoiesis [153, 154].

The discovery of thrombopoietin [155-162] (TPO; a cytokine which regulates platelet production) has enabled researchers to extensively study MK terminal maturation and proplatelet formation in vitro. When cultured in the presence of TPO, MK produce elaborate proplatelet extensions which ultimately release platelets in vitro thus, supporting the flow model of platelet production.

Spontaneous proplatelet formation of cultured mouse foetal liver MK was first described in depth by Italiano and colleagues (1999) [163]. Using real-time video-enhanced light microscopy, they observed terminally differentiated MK adhering to and spreading on substratum at a single cortical pole prior to generating an erosion site at

the opposite pole. As proplatelet formation was initiated, it was observed to gradually spread throughout the cell (the whole process taking ~10 hours).

Italiano *et al* also confirmed, prior to proplatelet formation, microtubules reside in the cytoplasm and radiate from the nucleus to the cell periphery. Such microtubule growth occurs at the plus ends by centrosome nucleation. Coinciding with this, MK adhere to the substrate through actin signalling. Dense pseudopodia structures protrude from the opposite pole to the adhered site and microtubules accumulate and bundle at the cortical region of such constructs. As pseudopodia structures extend and thin to form proplatelets, microtubules extend up through the cytoplasmic bridge and loop back at distal ends. The looping of microtubules forms a marginal band and thus, formation of nascent platelets (described in detail below).

Proplatelet growth occurs through two separate microtubule activities – continuous growth of plus ends and sliding of adjacent microtubules [164]. Microtubule dynamics during proplatelet formation were first described using a fluorescently tagged microtubule plus end-binding protein 3 (EB3) which fluoresces at microtubule nucleation/assemble sites. Initially microtubules assemble and extend from the centrosome at a rate ~10µm/min. As microtubule reach the cell cortex, they align parallel to the cell membrane. As proplatelet formation occurs, centrosome-mediated microtubule assembly ceases. Although microtubule polymerisation occurs throughout the proplatelet structure, proplatelet growth rate has been shown to be highly variable. The growth was also bidirectional suggesting proplatelet microtubules have mixed polarity. Furthermore, when inhibiting microtubule assembly (during proplatelet formation) with nocodazole, proplatelet elongation was not inhibited suggesting microtubule polymerisation is not the only force involved in proplatelet elongation.

Microtubule sliding is considered the primary force driving proplatelet elongation [165]. The molecule responsible for microtubule sliding is the minus end associated motor protein, dynein [164, 165]. Dynein permits microtubule sliding in an ATP-dependent manner. Inhibiting dynein activity through over expressing the dynamin subunit, inhibits proplatelet elongation. Furthermore, using FLAC time lapse and FRAP fluorescent imaging techniques to measure the rate of proplatelet elongation, it is apparent dynein dependent microtubule sliding is a dynamic process entering non-continuous extension, pause and retraction cycles; and under shear conditions the pause phase is shortened to enhance the rate of elongation.

Platelet components (e.g. organelles including granules, mitochondria etc) are individually transported by another motor protein, kinesin. Kinesin transports organelles bidirectionally along proplatelet microtubule bundles, at a speed of $\sim 0.2\mu\text{m}/\text{min}$, eventually becoming trapped in nascent platelets [166]. Kinesin moves directly over microtubules or by hitching a ride off sliding microtubules. MK also differentially package mRNA (messenger ribonucleic acid) and respective proteins into nascent platelets [167].

To increase the number of nascent platelets, proplatelets bend and bifurcate (branch) [163]. As proplatelets thin they form bead-like tandem arrays along the shaft which consist of protein and membrane. Although the role of these nodules is still unclear, it is hypothesised they are destined for platelet assembly following bifurcation. Although disrupting actin signalling has no effect on proplatelet growth, in its absence, bifurcation is inhibited [163]. Actin accumulates at the bent site and forms a network with tubulin to facilitate the branching process [168]. Bifurcation is dependent on non-muscle myosin IIA [169, 170]. Myosin IIA acts as a molecular motor which binds to

F-actin and provides the contraction force necessary for bending the proplatelet shaft. Contraction is regulated by small RhoA and its effector ROCK [171]. ROCK inhibits myosin phosphatase through phosphorylation of ser19 in myosin light chain 2 (MLC2) thus, promoting bifurcation [172]. An unbiased proteomic and polysome profiling approach has identified there are novel molecular switches which regulate proplatelet growth and bifurcation such as myristoylated alanine-rich c kinase substrate (MARCKS; a reversibly phosphorylated PKC) [173]. As MARCKS is reversibly phosphorylated it switches proplatelets between growth and bifurcation phases. Spectrin has also been shown to be essential for proplatelet formation [91]. When disrupting tetramer assembly IMS maturation and proplatelet formation no longer occur [91]. Furthermore, disruption during proplatelet formation destabilises and causes MK swelling and blebbing. In vitro proplatelet formation is fully described in Figure 1.5.

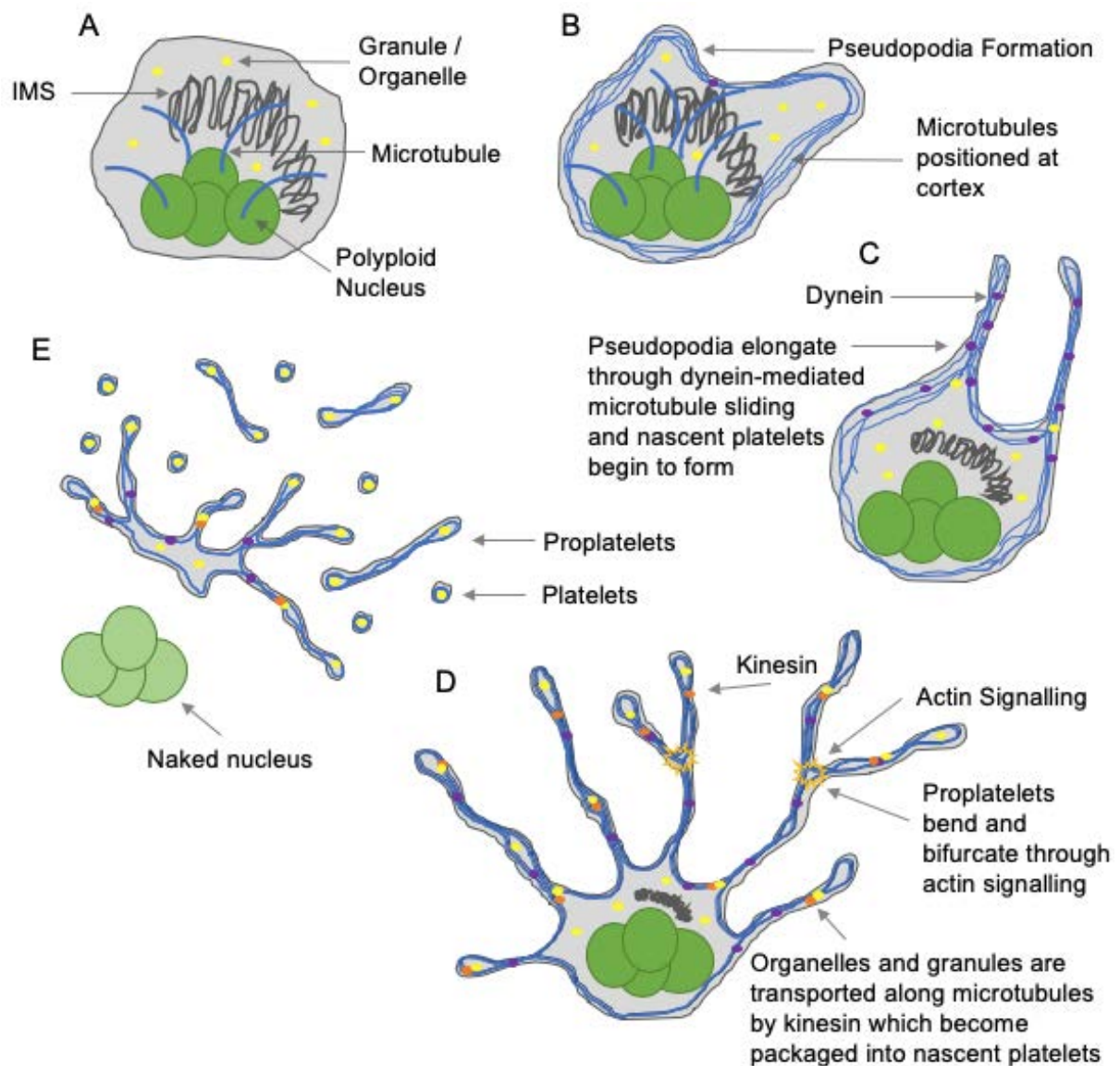


Figure 1.5: A Schematic of In Vitro Proplatelet Formation

Figure adapted from Platelets 4th Edition (2019). (A) As MK become mature, they develop a polyloid nucleus, an IMS and synthesise granules and organelles (yellow dots) for platelet biogenesis. Prior to forming cytoplasmic extensions, microtubules assemble and radiate from centrosome. (B) Microtubules become positioned and bundled at the cell cortex, assembly ceases and dense pseudopodia structures are formed at single pole through microtubule polymerisation. (C) As pseudopodia extend, microtubules dynamically polymerise and slide through dynein motor protein (purple dots) activation to form thin elongated proplatelet structures. Microtubules extend up from the cell mass through the proplatelet shaft and loop back at distal ends forming nascent platelet structures. (D) To amplify the number of nascent platelets, proplatelets bend and bifurcate through actin signalling. Prior to platelet release, kinesin (orange dots) traffick granules and organelles along microtubules which become captured and packaged in nascent platelets. (E) The entire MK cytoplasm is eventually transformed into proplatelet extensions, extruding the nucleus. Platelets and proplatelets are released from the MK mass. MK: Megakaryocyte; IMS: invaginated membrane system.

1.2.3 In Vivo Platelet Production

Key differences have been observed between in vitro and in vivo proplatelet formation. Junt and colleagues (2007) used a CD41-EYFP (enhanced yellow fluorescent protein) mouse model in which the EYFP was expressed as a target transgene of the gene locus for CD41 and multiphoton intravital microscopy to visualise thrombopoiesis in the BM of murine skulls [174]. MK were commonly found isolated and in close proximity to the sinusoid vessel. Unlike in vitro proplatelet formation where the entire MK becomes a mass of proplatelet extensions [163], in vivo MK reorganised their cytoplasm at the pole in contact with the vessel wall only. Furthermore, MK seemed to predominantly release large cytoplasmic structures and not terminally differentiated platelets into the bloodstream suggesting the final stages of thrombopoiesis continue within the vasculature downstream from the BM.

Shearing is also essential for in vivo proplatelet formation, the rate of extension and platelet release [175]. In addition to shear, other researchers have identified turbulence as a key factor in platelet biogenesis. The important role of turbulence was determined by Ito and others (2018) when imaging blood flow dynamics in the BM of EGFP mouse skulls using velocimetry and then recapitulating in an ex vivo flow-based bioreactor [176]. Results showed optimal turbulence activated platelet biogenesis and achieved maximal platelet shedding from proplatelets in the presence of combined high turbulence and shear.

The release of nascent platelets from proplatelet tips has never been observed in vivo. Recent evidence suggests, steady state platelet formation, prenatal and in adult life, occurs predominantly through MK budding rather than proplatelets. Realtime 4D imaging (3D + time) of adult BM, demonstrated ~2% MK formed proplatelets whereas,

84% released platelets via budding at a rate of 120 platelets per MK/h. Furthermore, sectioning of the major sources of BM in comparison with the spleen and lung showed at any given time ~1% MK were forming proplatelets whereas ~50% were budding. Given this, BM MK alone would form $\sim 3.4 \times 10^7$ platelets/h which equates to 89% of the estimated 3.82×10^7 platelets required to meet steady state demands. Interestingly, within the lung, albeit less, MK were observed and 20% of these formed proplatelets and released proplatelets structures into the bloodstream.

1.2.4 Sites of Platelet Release

1.2.4.1 Bone Marrow

Historically it is proposed MK maturation is spatially and temporally regulated by positive and negative cues within so-called BM niches (endosteal or osteoblastic and vascular). Proplatelet production is negatively regulated within the BM osteoblastic niche [177]. The osteoblastic niche is rich in collagen I and in vitro evidence supports that proplatelet formation is negatively regulated when MK are bound to this ECM component [178]. Ultimately, the association between collagen I and $\alpha_2\beta_1$ induces RhoA/ROCK signalling, which inhibits proplatelet formation through phosphorylating MLC2 [172].

In contrast, proplatelet formation is promoted in the vascular niche. When entering the final stages of maturation, it is thought MK undergo an SDF-1 (stromal-derived factor-1) mediated migration from the osteoblastic niche to the vascular niche (niche theory) where they associate with sinusoid micro-vessels [179-181]. Unlike the osteoblastic niche, the sinusoid niche is rich in collagen IV, VWF, fibrinogen, fibronectin and

laminin. As collagen I is absent in the blood vessel ECM, collagen IV/GPVI association alleviates its developmental constraints and promotes proplatelet production [182].

Due to advances in imaging, it is now possible to capture the BM architecture in its entirety. Using advanced microscopy, Stegner *et al* (2017) discovered the appearance of the BM is highly vascularised thus, challenging the so-called distant niche theory [183]. Instead of MK migrating from the osteoblastic niche it is proposed MK are situated in the sinusoid niche and are replenished by resident precursor cells. This new concept suggests, due to the proximity of vessels, MK are relatively immobile. Instead of promoting migration, SDF-1 potentially polarises MK and facilitates adhesion to the vessel. SDF-1 and fibroblast growth factor-4 (FGF-4) have been shown to enhance expression of vascular cell adhesion molecule-1 (VCAM-1) and very late antigen-4 (VLA-4) on sinusoidal EC, in which MK adhere to chemokine receptor-4 (CXCR4) promoting maturation and platelet biogenesis [179].

Various genetically modified mouse models support the notion that the ECM plays an important role in proplatelet formation. For example, mice which are VWF and GPIb β -null exhibit dysfunctional proplatelet formation [184]. As GPIB is an upstream regulator of RhoA and cdc42, disruption of GPIB or cdc42 also inhibits proplatelet formation [185]. In contrast in the absence of RhoA, cdc42 signalling is enhanced and GPIB-dependent transmigration of the entire MK occurs, suggesting GPIB-mediated cdc42 and RhoA signalling is a master regulator of proplatelet transendothelial migration.

Fibrinogen/ $\alpha_{IIb}\beta_3$ signalling has been shown to be important for initiating proplatelet formation. Larson and Watson (2006) discovered cultured primary mature murine MKs plated on fibrinogen produced a significantly higher number of proplatelets compared

to other matrices [186]. Furthermore, exposure to $\alpha_{IIb}\beta_3$ antagonists inhibited proplatelet formation signifying the regulatory nature of fibrinogen during proplatelet formation.

1.2.4.2 The Lung as a Site for Platelet Production

MK have been detected in peripheral blood of humans since the early 20th century [187], migrating as an entire entity from the BM through sinusoidal vessels [188]. However, although this phenomenon has been linked with haematological disorders, the physiological relevance was unknown [189-193].

Although some believe the lung is a dumping ground for MK-derived naked nuclei [194], others consider it a major contributing organ of platelet production. It was first suggested platelet production occurs in the lung by Howell and Donohue (1937) [187]. These researcher's discovered platelet counts in venous (left side) vessels of cats were higher than arterial (right side), suggesting the final stages of thrombopoiesis takes place in the lung. Since this finding, multiple researchers have agreed with this theory by observing the same results in several mammalian species [195-197]. However, predictions regarding contribution to the platelet count vary dramatically. Cell separation techniques such as elutriation have detected multi-nucleated MK with an intact cytoplasm in the lung which become lodged in microcapillary beds [198, 199]. As MK in venous vessels are incredibly rare it is thought that their trapping causes fragmentation in arterial vessels.

In support of lung platelet biogenesis, Junt and colleagues (2007) discovered when using intravital microscopy to visualise proplatelet formation in the BM of mouse craniums, the majority of platelet fragments released into the sinusoid microvascular

were larger than terminally differentiated platelets, suggesting shear forces within the lung arterioles play a role in thrombopoiesis [174]. The large cytoplasmic fragments released into the bloodstream are thought to circumvent platelet activation during their migration into the vascular space where they are retained in lung capillaries and fragment into platelets.

Using 2-photon intravital microscopy and a mouse strain which expresses EGFP positive MK and platelet membranes, Lefrançois and co-workers (2017) observed large MK migrating from the BM to the lung where they produced 10×10^6 platelets per hour (50% of the total platelet count) [200]. MK maturity in the lung was not only heterogeneous but haematopoietic progenitors were also present. This research group also observed under thrombocytopenic and stem cell deficient conditions, these progenitor cells migrated from the lung and repopulated MK in the BM, concluding the lung is a primary site of thrombopoiesis. From this research it is now recognised the lung contributes to thrombopoiesis however, the magnitude of this contribution is highly debatable. Recent 4D imaging of human adult BM, spleen and lung suggest the majority of MK are situated in BM (79%) however, 13% in spleen and 8% in lungs [201]. Thus, platelet biogenesis does occur in the lungs however, at least in humans, not to the extent observed within the BM.

1.2.5 Intrinsic Mediators of Megakaryocyte Maturation.

Changes in transcriptional profiles play a key role in MK proliferation and maturation. Such changes in gene expression, regulate MK lineage commitment from HSC and subsequent maturation events.

Conditional knockout of transcription factors GATA1, FLI1 , RUNX1, TAL-1 (or Scl) and NFE2 highlight their importance in various stages MK maturation (endomitosis, IMS development and proplatelet formation) [202, 203]. For example, mutations in GATA-1 (a zinc finger transcription factor) suppress MK endoreplication and IMS formation [203]. GATA-1 deficiency in murine MK causes an ~85% decrease in circulating platelets [204]. Platelets from these mice are spheroid and large. Furthermore, early stage megakaryopoiesis is dependent on GATA-1 and friend of GATA1 (FOG1) association. A single point mutation in the amino-terminal zinc finger of GATA-1 inhibits this association suppressing MK development [205]. Furthermore, FLI1 in cooperation with GATA1/FOG1 and ETS proto-oncogene 1 (ETS1) has been shown to drive expression of MK/platelet genes such as MPL, ITGA2B, GP2B and GP9 [205, 206]. In humans, mutations in GATA-1 give rise to multiple thrombocytopenic disorders with varied severities [207].

In addition to GATA-1, mutations in RUNX1 have proven to be detrimental for normal cytoplasmic maturation and ploidy. As in the latter, silencing of MYH10 by RUNX1 facilitates the transition from mitosis to endomitosis [147, 208].

TAL-1 (or SCL) is a GATA-1 target gene. Knock down of MK TAL-1 results in suppression of MK differentiation and development [209]. In the absence of TAL-1, expression CDKN1A is enhanced and polyploidisation is inhibited [210]. Importantly, this adverse effect is restored upon silencing CDKN1A. Overexpression of TAL-1 in human embryonic stem cells (HESC) has been shown to enhance MK and platelet production in vitro [211]. Furthermore, transduction of GATA-1, FLI1 and TAL-1 expression, results in enhanced MK differentiation from hPSC (human Pluripotent Stem Cells) [212].

In contrast, NFE2 activity is restricted to platelet biogenesis and not polyploidisation [213]. As MK reach full maturation, NFE2 is upregulated, which induces MK/platelet specific TUBB1 expression and subsequently promotes proplatelet formation [214, 215]. GATA1 and TAL-1 have been shown to transactivate NFE2 [213, 216-218]. When knocking down either NF-E2 and GATA1 transcription factors, MK lack β 1-tubulin and normal platelet production becomes dysfunctional. Unlike NF-E2 $-/-$ (where platelets are absent), GATA1 $-/-$ mice have low levels of circulating spheroid platelets consisting of a compromised marginal band [123]. Both GATA1 and β 1-tubulin $-/-$ platelets share a common abnormality of spherocytosis. However, only GATA1 $-/-$ platelet size is abnormal. This suggests, β 1-tubulin expression is indispensable during microtubule organisation and marginal band formation.

1.3 Platelet Lifespan and Clearance

Platelet turnover is a complex and tightly regulated process. To maintain normal haemostasis, a hundred billion platelets are released into and cleared from the bloodstream a day [146]. In conditions associated with enhanced platelet destruction, the majority of platelets are cleared through immune-mediated processes which are autoantibody (e.g., ITP (described in section “1.4 Thrombocytopenia”) or bacterial-induced sepsis). However, under normal physiology (without haemostatic stress or infection) only a small fraction of the total platelet population is consumed through haemostasis and the remaining surplus of platelets are cleared by other mechanisms.

Circulating platelets have a relatively short lifespan of 8-10 or 4-5 days in humans and mice respectively [6, 219]. This rapid lifespan is determined by two known

mechanisms: loss of sialic acids (desialylation) and/or programmed cell death (apoptosis) [220].

1.3.1 Intrinsic Apoptosis

Platelet lifespan is governed by the intrinsic apoptosis pathway. The importance of this pathway became apparent during BH3-mimetic pharmaceutical trials (ABT-737 and 263; a pro-death molecule) in oncology, when a major side effect was thrombocytopenia [221-224]. Pre-clinical investigations showed ABT-737 inhibits BCL-2, BCL-X_L and BCL-W pro-survival proteins and induced thrombocytopenia in mice and dogs through activation of pro-death proteins BAK and BAX triggering platelet apoptosis [225-227]. Furthermore, in the absence of BAK/BAX expression in mice, platelet lifespan doubles and this also prevents ABT-737-mediated platelet destruction [226]. From this study, BCL-X_L was identified as the key regulator of BAK and BAX [226].

The exact mechanism in which intrinsic apoptosis is triggered remains unclear. There are two theories thought to induce apoptosis: the “multiple hit” (damage inflicted during circulation) or “molecular clock” mechanisms (age related degradation of BCL-X_L) [35]. However, as BCL-X_L does not degrade constantly throughout the platelet lifespan this suggests an unknown mediator activates BAK and BAX directly [35].

BH3-only pro-death proteins (such as BID, BIM and BAD) become activated which sequester BCL-2 protein inhibition permitting BAK and BAX activity [228]. Mice lacking BAD show increased platelet lifespan however, it is unclear if this is due to a direct effect on BAK/BAX signalling [229]. Despite this, one proposed mechanism is BAD

downregulates protein kinase A (PKA) and permits apoptosis by sequestering BCL-X_L [230].

BAK/BAX activation induces mitochondrial degradation via caspase signalling [35, 231]. Like other cells, when undergoing apoptosis, PS is redistributed to the outer layer of the plasma membrane permitting phagocytosis and cell clearance. Unlike PS exposure during haemostasis, this redistribution is thought to be Ca²⁺ independent through Xk related protein 8 (Xkr8) [232, 233].

1.3.2 Glycan Mediated Clearance

Alternatively, to intrinsic apoptosis, loss of glycoprotein sialic acids has also been proven to be critical in regulating platelet lifespan and clearance. Glycan-mediated platelet clearance was first identified in vitro when Investigating why chilled stored platelets are rapidly cleared following transfusion [234]. These investigators discovered when exposing platelets to neuraminidase (a sialidase), this resulted in desialylation of platelet glycoproteins promoting the rapid clearance of platelets following transfusion. From these studies it became apparent platelets are cleared through two mechanisms: The Ashwell Morell receptor (AMR)/galactose association and resident liver macrophages [235-238]. Both mechanisms function through a common receptor, GPIb α [31].

The ligand binding domain (LBD) and mucin-like region of GPIb α consist of N-linked and O-linked glycans respectively [239]. The total carbohydrate composition of these glycans differs however, they both end with N-acetylglucosamine (GlcNAc) and galactose residues which are capped with sialic acid [31]. The loss of GPIb α sialic acid exposes surface galactose which associate with the AMR on liver resident hepatocytes

resulting in platelet clearance [240]. The AMR is a heterooligomeric lectin which consists of subunits ASGPR1 (HL-1) and ASGPR2 (HL-2) [241]. As senescent platelets enter the liver, they are detected by the AMR which triggers JAK2 (janus kinase 2) transducing subunit gp130-mediated STAT3 phosphorylation in hepatocytes [240]. Upon activation STAT3 is translocated to the nucleus where it translates TPO mRNA, ultimately regulating megakaryocyte differentiation and development and increasing de novo platelet production.

TPO is comprised of 332 amino acids and is heavily glycosylated. It binds to c-MPL (thrombopoietin receptor encoded by the MPL gene) which is expressed on HSCs, MK and platelets [242-245]. Serological concentrations of TPO inversely correlate with total peripheral platelet mass providing a regulated feedback mechanism to the BM for platelet production [246].

The specificity of TPO to the MK lineage is shown in mice which are deficient in TPO and c-MPL expression yielding 90% less platelets [247]. Although these mice show a dysfunction in TPO/cMPL signalling there is no observed effect on the production of other haematopoietic lineages. Hence, TPO/c-MPL signalling is the master regulator of MK development.

Despite a significant decrease in platelet production in TPO deficient disorders, platelets do persist, suggesting other mediators also influence platelet production. The colony stimulating factor (CSF), granulocyte and macrophage CSF (GM-CSF) and interleukin 3 (IL-3) play a role in in vitro MK proliferation [248]. Furthermore, such responses are also augmented in the presence of the IL-6 family of cytokines, IL-6, IL-11 and stem cell factor (SCF) and negatively regulated through erythropoietin (Epo) [249].

Alternatively to AMR-mediated clearance, *in vitro* studies have shown senescent platelets are also cleared through lectin-mediated processes [250]. During cold storage platelets can lose sialic acid and galactose exposing GlcNAc residues. Such carbohydrates are consequently recognised and cleared by hepatic macrophages through α M β 2 (Mac-1) integrin. The α M subunit contains a lectin binding site which binds to GlcNAc on β -glucan [251]. Interestingly, mice which lack the α M subunit have increased platelet counts suggesting α M β 2 also contributes to steady state lectin-mediated platelet clearance.

Neuraminidase is a glycoside hydrolase enzyme that cleaves the glycosidic linkages, removing sialic acid from adjacent galactose residues [252]. There are currently four known mammalian sialidases, neuraminidases 1-4 (neu-1-4) [253] but their role(s) in *in vivo* platelet ageing remains to be determined. However, investigations have shown in the presence of a sialidase inhibitors, the sialic acid of stored platelets are preserved and platelet survival is enhanced [254, 255]. Furthermore, bacterial infections induce neuraminidase production and therefore thrombocytopenia [235]. On platelets, neu-1 and 3 are stored in granular compartments or expressed at the PM, respectively. Neu 1 and 3 share a high affinity for sialic acid linkages within the mucin-like region of GPIb α . Desialylation of O-glycans within this region expose β -galactose residues which transmits a signal thus activating and unfolding GPIb α and its MSD, making the cleavage site for ADAM17 (ADAM metallopeptidase domain 17) accessible. Resting platelets also express β -galactose in granule compartments and presented on their surface [254]. Interestingly, the surface expression of β -galactose increases in stored platelets suggesting this mechanism determines β GlcNAc exposure and therefore, α M β 2-mediated macrophage clearance [31].

GPIb α is also constantly shed by ADAM17 on the surface of platelets throughout their lifecycle and the loss of this receptor is enhanced upon activation [256-258]. However, it is unclear if the rate of cleavage is equal on all platelets or whether the rate is constant throughout the platelet lifecycle.

Finally, ristocetin treatment of bacterial infection has been shown to induce VWF/GPIb α binding and upregulate BAK and BAX expression resulting in platelet apoptosis [259]. This suggests there is a possible link between platelet desialylation, apoptosis and clearance in which at least GPIb α is the central regulator.

1.4 Thrombocytopenia

Thrombocytopenia is classified as a platelet count below the normal reference range ($<150 \times 10^9/L$) [260]. These platelet disorders can either be acquired or inherited with highly variable aetiologies. A bleeding diathesis is often associated with a low platelet count [261-263]. However, many cases of thrombocytopenia have no bleeding defect and can go unnoticed throughout a person's life unless a routine blood test is required, or an individual suffers a severe haemorrhagic event.

Generally, acquired thrombocytopenia occurs because of impaired production, increased destruction of platelets or platelet sequestration in the spleen. Other common acquired conditions associated with decreased production are typically secondary to autoimmune disease (systemic lupus erythematosus (SLE)), cancer (myelodysplastic syndrome (MDS), aplastic anaemia (AA) or leukaemia), chronic infection (*Helicobacter pylori*, human immunodeficiency virus (HIV) or hepatitis C virus (HCV)) or in response to chemotherapy [264]. Table 1.2 shows examples of acquired thrombocytopenia and differences in rate of platelet production.

Table 1.2: Acquired Thrombocytopenia

ACQUIRED THROMBOCYTOPENIA	PLATELET PRODUCTION
Immune Thrombocytopenia (ITP)	Increased / Decreased
Thrombotic Thrombocytopenic Purpura/Haemolytic Uremic Syndrome (TTP-HUS)	Increased
Disseminated Intravascular Coagulation (DIC)	Increased
Drug-Induced (heparin and other)	Increased
Post-Transfusion / Transplant Purpura	Increased
Chemotherapy-Induced	Decreased
HIV-1	Decreased
Vitamin B12 and Folate Deficiency	Decreased

A single point gene mutation occurring during MK development and maturation can result in mild to severe thrombocytopenia. These rare inherited platelet disorders can be classified by inheritance patterns such as autosomal dominant, autosomal recessive and X-linked and/or by the size of platelets.

Inherited platelet disorders may have large or giant (macrothrombocytopaenia), small (microthrombocytopaenia) or normal platelets which can be identified by blood

counting and confirmed by a blood smear. Disorders associated with abnormal platelet size are termed congenital and cannot always be detected by current haematology analysers due to pre-set particle size limits. These conditions usually arise from MK cytoskeletal abnormalities resulting in dysfunctional proplatelet formation and platelet release. Congenital conditions are also characterised by abnormal platelet granules or tendency to form aggregates with other immune cells such as platelet/leukocyte aggregates (PLAs) or neutrophil Döhle-like bodies. Table 1.3 lists some examples of inherited thrombocytopenia with key clinical features and inheritance patterns.

Table 1.3: Inherited Thrombocytopenia

INHERITED THROMBOCYTOPENIA	GENE MUTATION	INHERITANCE	PLATELET SIZE
Congenital amegakaryocytic thrombocytopenia (CAMT)	MPL	AR	Normal
Thrombocytopenia with absent radii (TAR)	RBM8A	AR	Normal
Familial platelet disorder with predisposition to AML (FPD/AML)	RUNX1	AD	Normal
Radio-ulnar synostosis with amegakaryocytic thrombocytopenia (RUSAT)	HOXA11, MECOM	AD	Normal
Paris-Trousseau/Jacobsen syndrome (PT/JS)	FLI1	AR	Normal
Familial thrombocytopenia 2 (THC2)	ANKRD26	AD	Normal

Bernard-Soulier syndrome (BSS)	GPIBA, GPIBB, GPIX	AR or AD	Macro
Platelet-type von Willebrand disease	GPIBA	AD	Macro
TGA2B/ITGB3-related thrombocytopenia	ITGA2B or ITGB3	AR	Macro
MYH9-related disease (MYH9-RD)	MYH9	AD	Macro
TUBB1-related thrombocytopenia	TUBB1	AD	Macro
Filamin A mutations	FLNA	X-linked	Macro
DIAPH1-related thrombocytopenia	DIAPH1	AD	Macro
GATA-1	GATA1	X-linked	Macro
Gray platelet syndrome (GPS)	NBEAL2	AR or AD	Macro
Wiskott-Aldrich syndrome (WAS)	WAS	X-linked	Micro
X-linked thrombocytopenia (XLT)	WAS	X-linked	Micro

AR: Auto Recessive; AD: Auto Dominant; Macro: macrothrombocytopaenia; Micro: microthrombocytopaenia

BSS is an example of a well-known inherited macrothrombocytopaenia. This autosomal recessive disorder was first described in 1948 and is characterised by thrombocytopenia with giant platelets and a bleeding diathesis due to genetic mutations at alleles of GPIBA, GPIBB or GP9 genes which code for GPIb-IX. There are over 50 reported biallelic mutations affecting GPIb α , GPIb β or GP9 [265, 266].

These dual mutations cause inappropriate GPIb-IX/VWF signalling due to a reduction in GPIb-IX expression. As a result, platelet production and function are dysfunctional. Another receptor shown to be important in normal platelet production is $\alpha_{IIb}\beta_3$ [186]. Glanzmann Thrombasthenia (GT) is an autosomal recessive bleeding disorder associated by mutations in the ITGA2B or ITGB3 genes that express the subunits of $\alpha_{IIb}\beta_3$. Although GT is usually characterised with a defect in platelet function and not count, there have been several newly identified disorders associated with congenital macrothrombocytopaenia [39]. Many of the mutations are associated with the cytoplasmic salt bridge connecting the negatively charged β_3 -D723 and positively charged α_{IIb} -R995 proximal regions. [267-274]. These gain of function mutations induce $\alpha_{IIb}\beta_3$ to take on a highly activated conformation albeit with impaired surface expression. The formation of large platelets is a result of abnormal MK development and proplatelet formation supporting the notion $\alpha_{IIb}\beta_3$ plays an essential role in normal platelet production.

Alternatively, to dysfunctional receptor signalling, large platelets can arise from mutations associated with cytoskeletal proteins. MYH9-related disorders (MYH9-RD: May-Hegglin Anomaly, Sebastian Syndrome, Fechtner Syndrome and Epstein Syndrome) also result in macrothrombocytopaenia consisting of giant platelets (40% >3.9 μ m in diameter) [275, 276]. These disorders arise from various single point mutations of the MYH9 gene which encodes myosin IIA. Megakaryocyte development is normal in MYH9-RD however, proplatelet formation is defective [169, 277]. Dependent on the genetic variant (mutations in the c-terminal tail domain [278]) bleeding diatheses occur due to thrombocytopenia and dysfunctional clot formation [279].

1.4.1 Immune Thrombocytopenia

Immune thrombocytopenia purpura (ITP) is a common acquired form of thrombocytopenia and is classed as primary or secondary ITP [264, 280]. Primary ITP is characterised by a platelet count $<100 \times 10^9/L$ and commonly arises from a combination of antiplatelet-mediated processes which ultimately cause enhanced peripheral platelet destruction through innate or adaptive immune responses with increased or suppressed megakaryopoiesis [281-283]. In response to increased platelet destruction macrocytic stress platelets are produced [284].

ITP can occur in children and adults however, in children it is often acute whereas, in adults (particularly in >60's) ITP is chronic and persists for ≥ 12 months with an incidence of ~ 3 in 100,000 adults worldwide [285]. According to the 2019 NICE report, in the UK only, approximately 120 cases of ITP are diagnosed a year and 3000-4000 people suffer from ITP at any one time. Of these patients, two thirds have an associated bleeding diathesis (commonly of the dermis and mucosa layers) and clinically present a petechial rash (burst microcapillaries and blood pools under the skin presented as purple spots otherwise known as purpura haemorrhages).

Formerly known as idiopathic (an unknown spontaneous occurrence), ITP is now recognised as an autoimmune disease. It was first hypothesised there was an "antiplatelet" substance in the plasma of ITP patients by Harrington (1951) [286]. When injecting healthy individuals with ITP plasma he observed $\sim 60\%$ of individuals were inflicted with thrombocytopenia. This discovery led to the identification of antiplatelet IgG [287]. Plasma cell-derived autoantibodies are released in the blood and BM and commonly target platelet membrane glycoproteins ($\alpha_{IIb}\beta_3$ and GPIb α) [288-290]. Upon binding, autologous platelets are phagocytosed and cleared from the circulation

through the reticuloendothelial system by fc-dependent and -independent pathways [288, 291]. It is reported, 70-80% and 20% of cases are related to antibodies that recognise either $\alpha_{IIb}\beta_3$ or GPIb α respectively. However, rarer conditions are reported to have autoantibodies targeting both receptors or other platelet glycoproteins [292]. ITP patients have elevated levels of B-cell activating factor (BAFF) which causes B-cell proliferation and increased survival of B- and CD8 T-cells and secretion of interferon- γ (IFN- γ) [293]. Also, plasma cells residing in the spleen have been shown to be autoreactive and induce persistent disease [294]. Furthermore, autoantibodies from such plasma cells can suppress megakaryopoiesis [295].

Only ~60% of ITP cases are positive for autoantibodies. Other mechanisms involve abnormalities in T-cell responses. Compared to healthy subjects ITP patients have a high T-helper(Th)1/Th2 ratio which correlates with disease severity [296]. Furthermore, cytokine polymorphism affecting Th1/Th2, increase patient susceptibility [297]. This shift towards dysregulated immune activation is mirrored by a reduction in natural T-regulatory cells (nTreg) and increases in proinflammatory cytokines such as IFN- γ which suppresses immune tolerance. This lack of regulation can also result in Th1-mediated B-cell autoantibody production [298, 299]. De novo induction of induced Tregs (iTregs) in ITP modulates the T-cell immunogenicity. This shift in response was dependent on transforming growth factor- (TGF)- β 1-mediated tolerogenic dendritic cells (DCs) [300]. CD8 T-cells have also been shown to induce abnormalities in platelet production [301].

1.4.2 Platelet Clearance in Immune Thrombocytopenia

In ITP, platelet clearance generally occurs through an fc-dependent process [291]. The fc-dependent mechanism transpires when autoantibodies bind to platelet membrane glycoproteins in the bloodstream and upon entering the spleen, the fc region of platelet bound autoantibodies associate with fc γ -RIIa and fc γ -RIIIa on macrophages to trigger destruction.

First line IVIG (Intravenous immunoglobulin G) and anti-Rh(D) therapies block fc activity and have shown good efficacy in blocking fc-dependent platelet clearance [302]. Despite this, there are still patients who remain refractory to treatment and in the worse cases will require splenectomy. Such patients are predicted to have autoantibodies which target GPIb α proposing an alternative mechanism of platelet clearance in these individuals.

The fc-independent theory was identified by Li and colleagues (2013) [288]. This group discovered when injecting mice with $\alpha_{IIb}\beta_3$ or GPIb α monoclonal (Mab) f(Ab)2 (with absent fc region) antibodies, only the latter antibody induced platelet depletion suggesting there is an fc-independent mechanism which clears GPIb α -associated autologous platelets.

Following on from this work, Li and colleagues (2015) discovered that GPIb α platelet depletion was a consequence of glycoprotein receptor desialylation [303]. Human and mouse GPIb α and $\alpha_{IIb}\beta_3$ bound platelets were measured for galactose residues exposed following glycoprotein desialylation. GPIb α -treated human and mouse platelets demonstrated elevated receptor desialylation whilst $\alpha_{IIb}\beta_3$ -bound platelets did not. Whole blood analysis showed GPIb α antibody-bound platelets showed 20–60%

desialylation. Furthermore, anti-LBD antibodies have been shown to activate GPIb-IX, unfold the MSD and induce fc-independent platelet clearance [304].

T-cells can also enhance platelet clearance in ITP. With the help from CD4 T-helper cells, cytotoxic T-cells (CD8) can induce apoptosis in target cells. In ITP, in the absence of antiplatelet antibodies, CD8-induced platelet lysis and perforin/granzyme-mediated cytotoxicity is enhanced [305, 306].

1.4.3 Treating Immune Thrombocytopenia

ITP is categorised as newly diagnosed (diagnosed within 3 months), persistent (diagnosed 3-12 months previously) or chronic (>12 months ago). The decision to treat ITP depends predominantly on the severity of the disease such as platelet count and tendency to bleed. The primary goal of treatment is to improve patient quality of life by preventing bleeding, increase and stabilise the platelet count and therefore prevent chronic or relapsing disease. First line therapies include broad immunosuppressive corticosteroid drugs such as prednisone or dexamethasone where ~85% of patients tend to go into remission. However, even though the goal is to achieve optimal dosing, 50% relapse within 6 months and a further 25% by 12 months [307]. If steroids fail, immunoglobulins (such as IVIG – intravenous immunoglobulin; or anti-Rho(D)) can be administered. Although these therapies rapidly increase platelet counts, the response is transient and often associated with adverse effects [308, 309]. Thus, second-line therapies are often needed.

Second-line therapies broadly include splenectomy, rituximab (deplete B cells) and/or TPO mimetics (such as romiplostin or eltrombopag). Rituximab is a monoclonal CD20 antibody which binds to B cells and triggers depletion [310]. Rituximab can induce long-

term remission (5 years) in patients and some trials have published high remission rates in combination with other immunosuppressive drugs [311, 312]. However, not only is rituximab expensive, but issues such as impaired response to vaccinations and adverse side effects need to be considered. Alternatively, a splenectomy sees ~70% response, with 60% remaining in remission for >10 years. By removing the spleen, the main site of platelet destruction is removed. However, 30% of patients do not respond [307]. Although there are current tests (such as an autologous ¹¹¹In-labelled platelet scan) to determine the number of platelets trapped in the spleen [313], splenectomy is invasive, irreversible, and can increase the risk of thrombosis or infection and therefore, other treatments are often the first point of call.

The biggest advancement in recent ITP therapy is the use of TPO mimetics (romiplostim and eltrombopag). Instead of dampening the immune response (like all the above therapies), these drugs mimic TPO/MPL signalling and increase MK differentiation and development and subsequent platelet formation to compensate for enhanced platelet destruction [245]. Long-term studies (3-5 years) have shown both romiplostim (a peptibody given subcutaneously) and eltrombopag (non-peptide given orally) maintain platelet counts without toxicity [314, 315]. Other studies have shown good efficacy when combining with immunomodulating drugs [316]. However, 10% of people develop rebound thrombocytopenia following discontinuation, <10% develop bone marrow fibrosis and/or an increased risk of thrombosis (particularly in patients which may already be thrombogenic) [317].

1.5 Platelet Heterogeneity

Circulating platelets are heterogenous in age, size, density, function, metabolism. There are two concepts for this. The first is, young platelets are large and as they circulate, they lose granules and decrease in size, density and metabolic and functional capacity. Alternatively, heterogeneity is predetermined by release from different MK ploidy classes. Platelets from these different MK classes differ in physical properties e.g. RNA content. Despite this, the exact causes and significance of platelet heterogeneity remains debatable often yielding conflicting results. However, this, is likely due to differences in methodology regarding separation and measurements of platelet subpopulations.

1.5.1 Buoyant Density

Historically it was proposed that size and buoyant density are the defining factors of platelet immaturity [318]. Normal platelet density ranges between 1.04 to 1.09 g/mL, which is determined by granule content [319]. Early studies in humans by Karpatkin, utilising gradient density centrifugation, showed large platelets (rich in glycogen, orthophosphate and adenine nucleotide content) were more dense than small platelets [320]. These large platelets also contained greater metabolic potential and therefore, showed greater aggregatory potential [321]. Large dense platelets were also shown to contain greater mitochondrial enzyme activity suggesting large, dense platelets consisted of either a greater concentration of mitochondria than small low dense platelets or the same but larger [322]. In contrast, small, light platelets contained less granules than dense platelets [323] and displayed reduced sialic acid content [324]

suggesting the least dense were the oldest and loss of sialic acid was the mechanism to remove these platelets from the circulation.

Alternatively to platelets decreasing in density with age, it was proposed platelet density can also be determined by heterogeneity in MK ploidy class [325, 326]. Morphometric analyses confirmed that these differed in organelle content and concentration. Thus, comparison in density of the platelets derived from 8N or 32N MK demonstrated differences. However, these findings assume platelet size does not change in the circulation.

There are many discrepancies reported in the literature regarding platelet size and density and how they determine circulatory age. For example, in humans, platelet age and density positively correlate to maintain a constant density [327] whereas, in rabbits density decreases with age [320, 324]. Possible explanations for platelets becoming denser as they age is a constant accumulation of serotonin or acquisition of vesicles during their lifespan. Alternatively, it is suggested discrepancies between density and age are likely due to variations between species and continuous and dis-continuous density gradient methodologies [328]. Also, platelets may degranulate during gradient separation, thus skewing the overall density distribution.

1.5.2 Mean Platelet Volume

In addition to density, mean platelet volume (MPV) has also been thought to represent platelet age [329]. Platelet production is regulated to maintain a constant platelet mass. The platelet count and MPV determine the total platelet mass. Platelets with the highest MPV show the greatest affinity to aggregate however, little difference in density was observed between the largest and smallest platelets [330].

Advances in impedance techniques (incorporated into modern haematology analysers) have improved platelet sizing and therefore, made MPV routinely available in laboratories. However, this has led to issues regarding standardisation. Platelet parameters such as size are routinely measured in EDTA (ethylenediaminetetraacetic acid) anticoagulant. EDTA induces rapid spherocytosis in platelets [331]. Platelets spherizing in EDTA is induced by increases in cyclic AMP which permeates the membrane. As a result, platelets swell in a time dependent manner [332]. To overcome this, citrate anticoagulants are favoured as these maintain the discoid shape keeping a constant MPV.

Platelets are $\sim 2\text{-}3\mu\text{m}$ in diameter. This equates to an MPV of $\sim 8\text{-}10\text{fL}$. MPV has been shown to inversely correlate with the platelet count to maintain platelet mass. However, this is non-linear [333]. Once the platelet count surpasses $350\text{-}400 \times 10^9/\text{L}$ a plateau in the MPV is reached and therefore, the platelet mass increases also. This suggests there is a physical restriction regarding minimum platelet size. Despite this, MPV in conjunction with platelet count can be useful in diagnosing and managing some types of thrombocytopenia [334]. If the BM is intact with enhance peripheral destruction of platelets, the platelet count is low and MPV will increase. Alternatively, in the absence of BM production, platelet count and MPV are low. Thus, using MPV, it is possible to indirectly measure the rate of platelet formation. For example, in ITP associated with intact BM, as patients go into remission, platelet count and MPV will return to normal and upon relapse platelet counts decrease whereas the MPV increases.

Many factors determine platelet count and therefore, MPV. In experimentally induced thrombocytopenia, MPV can increase in as little as 4h whereas, shifts in MK ploidy occur ~ 2 days after. In contrast, in response to chronic stress such as in ITP, MK

ploidy classes shift and this incremental increase positively correlates with MPV [335]. It is hypothesised the regulation of normal MPV is governed by the average thickness of proplatelets. For example, canine platelets contain thicker and longer proplatelet processes than rats [336] with a respective MPV being 40% greater [337]. Also, IL-1 α has been shown to induce rapid MK fragmentation in response to acute thrombocytopenia [338]. However, MPV was not measured in response to IL-1 α injections. Thus, it is likely, rapid changes in MPV are a result from increases in MK cytoplasm and/or rapid induction of IL-1 α mediated MK fragmentation whereas, under persistent stress, shifts in MK modal ploidy maintain the formation of larger than normal platelets. In addition, it is now recognised MPV and platelet count are determined by genetic predisposition [339]. Furthermore, many of the genes identified are related to known forms of macrothrombocytopaenia.

Although it is inconclusive whether MPV is determined by MK heterogeneity and/or platelet ageing, platelet size has been shown to be clinically useful. For example, in thrombocytopenia associated with enhanced platelet turnover or suppressed BM production, patients with low platelet counts but with increased MPV have less frequent bleeding episodes. Agreeing with previous findings, large platelets have greater thrombotic potential [340]. Furthermore, MPV is now recognised as a potential biomarker for identifying cardiovascular disease and increased risk of thrombosis [341, 342].

Despite such findings, using MPV to determine platelet age, assumes all young platelets are large. Although this is generally true, during steady state production it is apparent size does not necessarily determine age [327, 343]. However, this does not account for rapid changes in size which may occur following MK release to the point

when MPV is measured. In contrast, increases in plasma levels of TPO significantly decrease platelet size in healthy human subjects [344] and this is reversed when severe platelet loss is inflicted in platelet disorders such as ITP [345] or during BM recovery post chemotherapy [346]. Furthermore, in ITP and inherited macrothrombocytopaenia, not all large platelets will be young. MPV also fails to discriminate between BM failure patients and normal [347]. As a result, the rate of platelet formation is often overexaggerated. Also, lack of standardisation between haematology analysers mean abnormally large platelets are often excluded from the size distribution [348].

As it can be challenging to define young platelets by size, age can also be categorised by phenotype. For example, young platelets are better known as reticulated platelets (RP) [349] and old or mature platelets are determined by expression of apoptotic factors or by senescence and desialylation [35, 236, 350, 351].

1.5.3 Reticulated Platelets

RP (also known as immature, young or newly formed platelets) were first described by Ingram and Coopersmith (1969) [349] whilst investigating platelet production in a canine model of acute blood loss. Using new methylene blue labelling of blood films (a method commonly used to identify reticulocytes) these researchers observed in response to daily bleeding a new type of platelet which contained a dense reticulum and was greater in volume compared to non-labelled platelets. The cellular material which is stained intravitaly with new methylene blue is RNA and rough endoplasmic reticulum thus, RP also appeared to have increased levels of RNA analogous to erythroid reticulocytes. Furthermore, electron microscopy of immature platelets

demonstrates RP contain an abundance of ribosomes, rough endoplasmic reticulum and remnants of golgi apparatus compared to normal circulating platelets [352].

Although the new methylene blue method was capable of measuring RP, it was unsuitable for rapid measurement and ultimate translation to clinical use. To overcome this, a flow cytometry method was designed to measure RP in whole blood [353]. As RP were proposed to be equivalent to reticulocytes, Kienast and Schmitz (1990) combined conventional flow cytometry (CFC) with an established reticulocyte fluorescence dye, thiazole orange (TO) labelling RNA [354] to discriminate RP from mature platelets. TO permeates the cell membrane, binds to nucleic acids and exhibits a maximum absorption at 475 nm and emits a maximum fluorescence at 530 nm . As RP are hypothesised to be large and have an increased RNA content compared to mature cells, they were discriminated by forward scattered light (FSC) and fluorescence intensity (measurement highly dependent on platelet size and TO signal). Thus, this method assumes all newly formed platelets are large.

1.5.4 The Clinical Relevance of Reticulated Platelets

Using in vivo biotinylation of platelets in mice, researchers have confirmed that RP are indeed the youngest circulating platelets with a short lifespan of 12 hours to 1.8 days (which appears to differ between species). In analogy with reticulocytes, measurement of circulating RP therefore has the potential to measure the rate of platelet production or thrombopoiesis [5, 355, 356]. This is confirmed in thrombocytopenic conditions associated with increased platelet production with a greater number of RP compared to healthy blood [355, 357, 358]. RP measurement therefore has clinical potential the classification and management of thrombocytopenia.

Although several researchers have shown RP can identify and monitor certain thrombocytopenic conditions, the specificity of TO labelling has come into scrutiny. Researchers have reported the percentage of TO stained platelets positively correlates and increases with incubation time and dye concentration [353]. This suggests that the RP TO labelling is not saturable and that non-specific labelling of nucleotides within the dense granules is occurring [346, 359, 360]. This was confirmed in patients with dense granule defects (i.e. Hermansky Pudlack syndrome or storage pool defects) displaying significantly lower TO signals [360]. In addition, other methodological variations were also apparent such as preanalytical variables, sample handling (whole blood or platelet rich plasma (PRP)), effects of fixation and various gating threshold settings and strategies (reviewed in [361]). Although the method has shown some utility, standardization remains a significant obstacle to the method having clinical utility.

Despite such issues, an automated RP measurement (called the immature platelet fraction; IPF) was incorporated into haematology analysers such as in XE and then more recently the XN series by Sysmex, Kobe (Japan) [362-364]. This standardised method uses innovative nucleic acid dyes (a mixture of polymethine and oxazine dyes (Fluorocell Platelet®)) to discriminate platelets from RBC (red blood cells) using flow cytometry principles. Platelets are quantified in the Platelet-Fluorescence channel (PLT-F). In this channel, Fluorocell Platelet® labels nucleic acid in platelets and is excited by the 633nm laser. In combination with forward scattered (FSC) light (size) the total platelet count is accurately quantified. From this measurement, the largest platelets with the greatest Fluorocell Platelet® signal determined by a systematic designed algorithm) are classified as the IPF (Immature Platelet Fraction).

Unlike RP measurement, IPF measurement is a rapid, automated, standardized, and has shown good reproducibility both between and within laboratories [364]. Furthermore, the RP measurement in healthy controls showed a wide normal range (1-15%) whereas, the IPF range was narrower 1.1 – 6.1% with a mean of 3.4% [361]. Although RP and IPF values correlate however, due to non-specific labelling, the percentage of RP is generally higher.

The only other instrument capable of measuring RP is the CELL-DYN Sapphire (Abbott Diagnostics, Chicago, IL, USA; termed retPLT) [365]. Platelets are discriminated from RBC using 3 angles of scatter light plus fluorescence emitted from a propriety dye (CD4K530, excited by the 488nm laser). Programmed algorithms differentiate retPLTs from other platelets by CD4K530 and the 7° scattered light.

When comparing RP measured by conventional flow cytometry and IPF, various studies have reported good correlations in thrombocytopenia with enhanced peripheral destruction [366, 367]. However, from these studies, little to no correlation was reported in healthy controls [367, 368]. Furthermore, direct comparisons between IPF and retPLT show weak to moderate correlations [369]. One explanation for such disparities is dye specificity. As mentioned above TO binds to dense granules and mitochondrial RNA whereas, Fluorocell Platelet® has been proven to preferential bind to mitochondrial RNA only [370]. Furthermore, retPLT is adjusted for size. Together this suggests, although these methods provide some information regarding thrombopoiesis, they appear to reflect different aspects of platelet production and are therefore limited in their use within a clinical setting.

Considering such findings, alternative dyes have been proposed such as SYTO 13 (ThermoFisher). SYTO 13 is a nucleic acid dye which has a high affinity to RNA where

its fluorescence is enhanced following binding. This nucleic acid dye is cell permeant with an absorption maximum of 491nm and an emission maximum of 514nm. SYTO 13 not only exhibits a greater quantum yield than TO, [371] but its fluorescence stability remained for at least 5h following labelling for 90 min. Importantly, in healthy individuals, SYTO 13 positively correlates with IPF ($r = 0.668$) suggesting similarities between the dyes. In contrast, its relationship with TO labelling was moderate ($r = 0.478$). Furthermore, unlike other nucleic acid dyes, SYTO 13 labelling can be used to sort RNA rich and low platelet populations offering good technical advantages. Recently, sorting RP by SYTO 13 expression in conjunction with transmission electron microscopy has shown RP are larger, more granulated and contain a greater number of mitochondria than non-RP [372].

Another marker shown to recently indicate platelet age is human leukocyte antigen I (HLA I)/major histocompatibility I (MHC I) [371, 373]. MHC I is expressed by all nucleated cells and play a vital role in presenting antigen to T cells to trigger an immune response to viral infections. Such responses have also been reported in platelets [374]. When investigating mechanisms of anti-HLA I alloimmunisation following transfusion of platelets in experimental mouse models, Angénieux and co-workers (2019) observed a subpopulation of platelets which displayed enhanced HLA I expression [373]. This same group showed, TO-bright platelets displayed greater HLA I expression which is rapidly shed within the first 24h in circulation. Furthermore, this TO_{-bright}/HLA I_{-high} subpopulation was shown in humans and importantly could be used to indirectly determine the rate of platelet production in control and ITP blood. As HLA I labelling is antigen specific, the authors propose this method to be more superior to RP and IPF in the classification of thrombocytopenia. In contrast, it is thought that

platelets can also acquire soluble denatured HLA I protein from the circulation during their lifespan [375-379] thus questioning whether this is a suitable marker for young platelets.

1.5.5 Preplatelets

Although our understanding of platelet production has improved significantly over recent decades, details of the final stages of platelet biogenesis remain controversial and elusive. Although the consensus view was that BM MK release platelets in a terminally differentiated state, [151, 152] there was historical evidence that MK and/or proplatelets could also circulate in the bloodstream and release platelets in the pulmonary circulation [187]. However, due to advances in imaging techniques and the availability of recombinant TPO, terminally matured MK metamorphose into proplatelet structures that can then release platelets *in vitro*. Furthermore, *in vivo* labelling of BM MK demonstrated that they could also release large cytoplasmic structures or proplatelets into the sinusoid vessels suggesting that platelet maturation events continue within the bloodstream [163, 174, 380, 381] and the pulmonary circulation [187, 200]. Furthermore, circulating proplatelet-like structures have been observed in rats [381].

Considering this, Schwertz *et al* (2010) observed platelets incubated in serum-free M199 media at 37°C for 6h could undergo transformation into barbell platelets [382]. Quantification by microscopy suggested this occurred in a subpopulation of platelets (~5%). By capturing solitary platelets in micro-droplets for a maximum of 6h, it was confirmed single platelets formed barbell structures which eventually underwent fission to form two smaller platelets or “progeny”. Such progeny were ultra-structurally similar

to and functionally the same as blood platelets with respiring mitochondria. Moreover, during barbell formation, platelets exhibited increases in biomass, cellular protein levels, protein synthesis and mitochondrial DNA (deoxyribonucleic acid) replication. Thus, strongly supporting the notion, platelet formation continues following MK release.

The same year, Thon and co-workers (2010) also observed platelets undergoing fission within a murine foetal liver MK culture system [380]. This group described a new terminal stage of platelet maturation through a platelet intermediate, the “preplatelet”. Preplatelets are large (3-10 μ m in diameter), discoid cells which can reversibly interconvert into barbell platelets and undergo fission to form two smaller platelets (Figure 1.4). The majority of preplatelets were shown to be 3-4 μ m in diameter whereas, in rare cases 4-10 μ m was observed.

Electron microscopy shows the largest preplatelets contain enough platelet components such as invaginated membranes, α -granules and mitochondria to form 6-20 terminally differentiated platelets through continuous rounds of fission [380]. Furthermore, preplatelets were shown to contain multivesicular bodies further supporting the notion that protein synthesis may occur during preplatelet/barbell conversion.

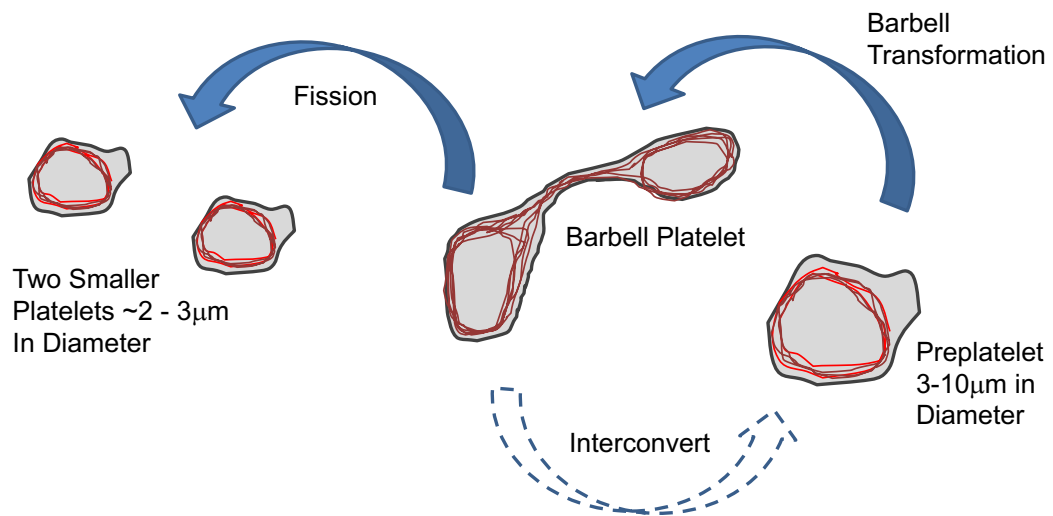


Figure 1.6: Preplatelet Maturation

Preplatelet/barbell conversion is considered the final stage of platelet maturation which continues in the bloodstream. Circulating preplatelets are large platelet intermediates (3-10µm in diameter) which are released by MK proplatelets via budding. These large platelet precursor cells can interconvert into barbell platelets by reorganising their microtubules into a continuous figure-of-eight morphology forming a thin cytoplasmic bridge interconnected by two nascent platelet structures containing constructed marginal bands (resembling those during proplatelet formation). To complete maturation, preplatelet microtubules twist recurrently at the midsection and then undergo fission into two smaller platelets (2-3µm in diameter).

Visualisation of microtubule dynamics during this transformation showed preplatelets elongate and twist recurrently at the midsection forming two platelet tear-drop structures interconnected by a cytoplasmic bridge characterised by a continuous figure-of-eight marginal band morphology. During this process microtubules polymerise and dynamically assemble as the marginal band reorganises prior to completing fission [380]. During this process, granules are transported along tubulin shafts and packages into nascent platelets. Furthermore, Mice heterozygous for inactivated Pi3K(phosphatidylinositol 3 kinase)-C2a on platelets displayed abundant levels of circulating barbells [383]. Heterozygous inactivation of Pi3K-C2a resulted in a significant reduction in the basal pool of Pi3P (phosphatidylinositol 3 phosphate) and

therefore, mislocalisation of several key membrane proteins including spectrin, myosin, FLN and moesin suggesting an important role for Pi3K-C2a in membrane remodelling during barbell conversion.

Thon *et al* (2012) quantified preplatelets and barbells in human platelet rich plasma (PRP) using a laser scanning cytometry technique [384]. Using this method it was proposed ~3.6% of circulating platelets are preplatelets suggesting they may represent IPF in healthy individuals [363]. Furthermore, barbells were rare events (0.05%), suggesting preplatelet/barbell transformation is transient.

Preservation of barbells in PRP was anticoagulant-dependent, where barbells were present in trisodium citrate and AJ buffer but absent in EDTA and heparin [384]. This may explain why barbells are not frequently observed during routine blood smears generated from EDTA whole blood. Upon exposing barbells to EDTA or heparin they are converted back into sphered preplatelets. β 1-tubulin fluorescent labelling showed preplatelets in EDTA and heparin consisted of a smaller but denser marginal band characterised by intense fluorescent labelling. This suggested marginal band thickness is an important determinant of barbell conversion.

From this, the same group mathematically modelled preplatelet/barbell conversion based on peripheral microtubule number and diameter [384]. This model predicts the thickness of the platelet marginal band (or microtubule bundle) negatively correlates with circumference. Thus, larger, thinner marginal bands can overcome microtubule elastic bending and actomyosin-spectrin compression forces to facilitate barbell transformation. As preplatelets undergo maturation they decrease in size and microtubule bundles become too thick to overcome such forces. This suggests, preplatelet maturation regulates terminal platelet size. Furthermore, when incubating

platelets from FLNA deficient mice (characterised by dysfunctional proplatelet formation and macro-platelets with greater numbers of cortical microtubule bundles) barbells were deformed suggesting that some macrothrombocytopaenia disorders could be due to a dysfunction in preplatelet maturation [384, 385].

Although the above studies suggest preplatelet maturation is restricted to a subpopulation of platelets (i.e. RP), the physiological importance in humans has yet to be fully defined. However, when isolating proplatelets from a murine foetal liver MK culture system and transfusing them into wild type (WT) mice, platelet counts increased 300% more than a standard platelet transfusion [380]. In vitro analysis showed when exposing preplatelets to shear forces, conversion into barbells is enhanced. Furthermore, extended incubations have shown platelets form barbells which also increases platelet counts [382]. Together this suggests, preplatelet maturation is an important process which takes place in the bloodstream and contributes to the total platelet count.

The discovery of preplatelet maturation has raised many questions. Is preplatelet maturation an event which takes place in the bloodstream and if so, is it restricted within a subpopulation of platelets such as immature platelets. Although quantification of preplatelets and barbells in PRP suggests they are analogous to immature platelets this has not been proven. Furthermore, could their quantification be of potential clinical utility in the diagnosis, classification and management of both acquired and inherited thrombocytopenia.

1.6 Image Flow Cytometry

It is thought that preplatelets in common with RP also lack specific immunological markers to completely discriminate them from other platelets. Therefore, in order to detect both preplatelets and their barbell intermediates, the use of new high throughput image-based methods will be essential for their characterisation and accurate quantification in whole blood.

CFC is an invaluable scientific tool used for investigating platelet phenotype, function and production, which has led to the theory platelets exist in subpopulations formed during thrombopoiesis or induced by local milieu determined by physiological state. However, the caveat of CFC is it lacks any image content including morphological depiction and compartmentalisation of fluorescent spatial distribution and therefore often yields inaccurate measurements due to high coincidence and poor signal to noise ratios. Conversely, although microscopy can offer high content resolution this technique is time consuming and highly subjective.

Imaging flow cytometry (IFC) combines the speed, sensitivity and phenotyping abilities of CFC with the single cell image content resolution afforded by microscopy achieving high throughput morphological and spatial measurements [386, 387]. Such high throughput quantification means it is possible to use IFC to identify and quantify immunologically similar cell populations and subsets; maturation events and phenotypes; and cellular interactions which would otherwise be challenging by CFC [388-390]. Furthermore, with advanced morphometric spatial depiction, IFC has the potential to replace laborious microscopy-based clinical techniques such as blood smears [391-395].

The ImageStream^x-MKII (ImageStream^x, Luminex Corp, Texas, USA) is the most recent commercially available multispectral IFC [387, 396]. Hydrodynamic focussed cells are trans-illuminated by brightfield (BF), orthogonal lasers (options: 405, 488, 561, 594, 642 or 785nm) and side scattered light (SSC or darkfield). Emitted light is collected by a high-resolution objective lens (x20 - x60) and relayed to a dichroic filter stack. Light from the stack is organised into spectral bands to generate images of cells which are projected on a CCD (charge-couple device) camera. (further details regarding instrumentation specification, operation and software can be found in section 2.8 “General Methods”).

The ImageStream^x uses a robust image analysis platform called Image Data Exploration Analysis Software (IDEAS) [386, 397]. IDEAS generates pixel masks and features to score and discriminate cells of interest based on quantitative morphological metrics such as size, shape, texture and intensity (see section 2.8 “General Methods” for complete description of masks and features). Systematically designed masks are used to portray spatial characteristics within a single cell and to quantify object-based measurements whereas, features are used to conduct morphometric measurements base on brightfield, SSC and/or fluorescence within the cellular compartments defined by the masks [390].

Since the development of ImageStream^x, it has been used in a multitude of scientific fields including, oncology, cellular function and development, clinical identification of established and onset of disease, vesicular biology and oceanography; displaying good efficacy and great potential to be translated into a clinical setting [398-414]. In platelet biology, IFC has accurately and precisely defined platelet leukocyte

interactions; platelet-derived vesicle characterisation, association and function; and platelet activation, production and clearance [304, 405, 408, 415-417].

The ability to accurately characterise and quantify platelet production and turnover is of great use clinically to improve diagnosis and management of platelet disorders. ImageStream^X has shown good efficacy in identifying rare normal and abnormal MK maturation events in human BM. As MK are rare myeloid cells accounting for <0.1% of all cells in the BM, phenotypic quantification of maturation events is highly challenging and ultimately CFC measurements are often exaggerated, or alternatively rare events are missed. CD41 and DRAQ5 (nuclei) are markers commonly used to immunophenotype MK lineage commitment by CFC. However, by fluorescently labelling MKs isolated from the BM of murine femurs with such markers and comparing CFC quantification of maturation events with ImageStream^X, Niswander and co-workers (2015) showed >50% of MK events measured by CFC artefacts containing CD41 tethered platelets to non-MK cellular events [416]. Furthermore, the majority (70%) of true MK events consisted of >1 MK leaving only 15% of the total being legitimately single events. To accurately identify primary MKs by IFC, Niswander designed multiple morphological measurements based on DRAQ5 (nuclear dye) intensity and CD41 and CD9 object masks. From this, they were able to accurately determine MK ploidy classes. This approach permits quantification and therefore, frequency of MK maturation events.

IFC has also been used to improve current CFC methodologies to determine platelet leukocyte aggregates (PLA; including monocyte, neutrophil and lymphocyte subsets). PLA are a characteristic endpoint of platelet activation in whole blood. Circulating PLAs are elevated in cardiovascular disease and are considered a biomarker of in vivo

platelet activation and cardiovascular risk [418]. Although PLA's can be measured by CFC, the sheer magnitude of platelets compared to leukocytes can also result in measurement of untethered platelet/leukocyte coincidence events if not controlled for by assay optimization (e.g. final dilution and flow rates).

Linden and colleagues have shown that ImageStream^X is a superior discriminator PLA compared to CFC yielding a lower number of actual tethered PLA with an increase in reported coincidence events that can be imaged [415]. Furthermore, using ImageStream^X, PLA have been shown to play a critical role in chronic inflammation and pulmonary dysfunction in sickle cell disease.

1.7 Hypotheses and Aims

The central hypotheses of this thesis are:

- 1) Preplatelets represent circulating immature platelets and can divide to undergo terminal maturation to maintain physiological platelet counts.
- 2) Quantification of preplatelets and their circulating barbells will provide a new clinical assay and provide deeper insight into the kinetics and pathology of thrombocytopenia.

Therefore, the objectives of this work were as follows:

- 1) To set up and evaluate a new method for accurately and rapidly quantitating the number of preplatelets and barbell platelets within human and mouse normal

and pathological blood samples using alternative temperatures and anticoagulants (e.g. citrate) to EDTA (Chapters 3 and 4).

- 2) Accurately characterise and quantify preplatelet maturation events in normal human blood samples using advanced microscopic and ImageStream^X imaging techniques (Chapter 4).
- 3) Determine if preplatelets are indeed immature platelets and if so, establish the physiological importance of platelet maturation in whole blood. Furthermore, investigate whether quantitation of such events offers a new clinical assay to provide deeper insights into platelet kinetics and the pathology and various clinical conditions where platelet formation is either enhanced or decreased (Chapter 5).
- 4) To determine preplatelet maturation kinetics in normal and experimentally induced thrombocytopenia in mice. Use in vivo biotinylation to definitively confirm whether preplatelets and their barbell derivatives are equivalent to newly formed immature platelets (Chapter 6).

Chapter 2 - General Methods

2.1 Study Recruitment

2.1.1 Ethical Approval and Study Management

The clinical study was authorised by Research and Development (R&D; reference: RRK 5677) and the Integrated Research Application System (IRAS) ID: 188445. Recruitment of healthy volunteers and acquired thrombocytopenia patient cohorts was approved by the National Health Service Research and Ethics Committee (NHS REC; reference: 15/WM/0465), sponsored by University Birmingham (UOB) and took place at the University Hospital Birmingham (UHB) NHS Foundation Trust. Study management was conducted through the National Institute for Health and Research (NIHR) Clinical Research Network's (CRN) Central Portfolio and Management (CPMS) system (CPMS ID 39481). Using CPMS, all research activity was regularly updated and submitted.

2.1.2 Patient Cohorts

Participants consisted of males and females with an age range of 18 - 82. Patient cohorts were allocated into three groups:

- 1) Normal Healthy Controls
- 2) Primary ITP and
- 3) Myeloma or high-grade lymphoma patients receiving chemotherapy prior to an autograft stem cell transplant.

Healthy volunteers were recruited from the University of Birmingham, Centre for Translational Inflammation Research (CTIR), New Queen Elizabeth Hospital (NQE) and Institute of Biological Research (IBR). Acquired thrombocytopenic patient cohorts were recruited from the Centre of Clinical Haematology (CCH) and oncology ward

QEHb respectively in collaboration Dr. Gillian Lowe (consultant in haematology) and Dr. Pip Nicholson (registrar in clinical haematology),

Primary, chronic ITP patients donated blood on a one-time basis only and consisted of males and females with mixed UK ethnicities, an age range between 18 and 80 and were either receiving no medication (n=3) or (all other patients) varied daily or weekly doses of prednisone and/or a TPO mimetic (romiplostim or eltrombopag). ITP platelet counts, MPV and IPF% can be viewed in Chapter 5, Figure 5.3. In contrast, myeloma or lymphoma patients were receiving chemotherapy which consisted of a single high dose of melphalan or six days of LEAM (Lomustine, Etoposide, cytarabine and low dose Melphalan) followed by an autograft stem cell transplantation on the subsequent day post therapy. Blood samples were taken before chemotherapy (i.e. at baseline) and 6 days following the autograft transplant (i.e. at the platelet count nadir: with no platelet production). To maintain confidentiality, donor and subsequent specimens were given an anonymised code.

Patients were excluded from the study if thrombocytopenia was caused by an alternative condition to ITP, patients had a suspected congenital disorder, received a blood transfusion within 7 days prior to recruitment, were currently receiving treatment known to affect platelet function and/or diagnosed with severe anaemia.

2.2 Mice

All mice were bred and housed in the Biomedical Services Unit (BMSU), University Birmingham. Within this facility, mice were bred and reared in individually ventilated cages (IVCs) under strictly regulated environmental conditions including temperature

(16-26°C), humidity (40-60%), Light intensity and cycle (14h light 10h dark and diet (~6% fat).

WT C57/6 mice were obtained from an in-house breeding colony and were confirmed to be without bleeding defects via in house genotyping procedures. All animal procedures were undertaken with the UK Home Office approval in accordance with the Animal (Scientific Procedures) Act of 1986 working under project licence number P46252127 held by Professor Yotis Senis from the institute of Cardiovascular Sciences, University of Birmingham.

2.3 Phlebotomy

Human blood samples were collected by venepuncture from the median ante-cubital vein which lies over the cubital fossa or the basilic vein which runs over the back of the wrist and hand. Using a 21G Butterfly® needle with a Safety-Lok blood collection set (Becton Dickinson (BD), #368654, New Jersey, USA), blood was drawn directly into either 1/10th volume of 3.2% 0.105M trisodium citrate (citrate 4.5mL; BD, 367691), lyophilised 7.2mg K₂E EDTA (4mL; BD, 367839) or recombinant hirudin (3.5mL; Roche, #08128812001, Basel, Switzerland) sterile vacutainer®(s). blood was processed immediately or within 1 hour post phlebotomy.

Murine blood samples were taken via tail bleed or terminal/cardiac bleed via the inferior caudal vena cava under isoflurane anaesthesia. For tail bleeds, the tail vein was pricked with a 23G Microlance™ needle (BD, #237403) and blood was milked by applying pressure at the base of the tail with the tip of a finger and thumb and repeatedly and smoothly running down to the tip of the tail. Blood droplets were

collected into a 1.5mL Eppendorf containing 20 μ L of EDTA (5mM; Sigma, #E5134, Missouri, USA) or 4% citrate (Sigma, #S5770). For terminal bleeding, blood was aspirated with a 23G MicrolanceTM needle into a 1mL syringe and transferred immediately into an Eppendorf containing 100 μ L of EDTA or 4% citrate. All blood samples were mixed with respective anticoagulants by gently inverting the collection tube 6 times. Eppendorf's were weighed with anticoagulant only and anticoagulant plus blood to determine the total blood volume of each sample and appropriate dilution factors were calculated.

2.4 Platelet Preparations

2.4.1 Platelet Rich Plasma (PRP)

Human anticoagulated whole blood was collected from all patient cohorts as above and centrifuged at 200g for 10 minutes at 21 or 37°C. Following centrifugation, RBCs were pelleted and separated from the PRP by a thin white leukocyte-rich buffy coat layer. To ensure large platelet structures were included in all analyses, PRP was extracted down to 2mm above the buffy coat and carefully transferred to a sterile 5mL falcon tube without disturbing the buffy coat. PRP was used for incubation assays, immunofluorescence techniques and aggregometry.

2.4.2 Platelet Poor Plasma (PPP)

Following PRP generation, 1 μ M of prostacyclin (or prostaglandin I₂: PGI₂; Cayman Chemicals, Michigan, USA, #61849-14-7) was added to PRP and centrifuged at 2000g for 20 minutes at 4°C. Once platelets were pelleted the supernatant was transferred into a 1.5mL Eppendorf and centrifuge at 13,000g for 2 minutes at 4°C. The final

supernatant or PPP was transferred into a new 1.5mL Eppendorf and either used immediately or stored long-term at -80°C. PPP was used as a negative control for platelet aggregometry.

2.4.3 Washing Platelets

Anticoagulated whole blood was collected from all patient cohorts as above. Immediately after venepuncture, warmed (37°C) acid citrate dextrose (ACD; sodium citrate 85 μ M, glucose 110 μ M & citric acid 71 μ M) was added (1:10 dilution) to the whole blood, mixed by gently inverting the blood tube 6 times and centrifuged at 200g for 10 minutes at room temperature (see PRP). Following centrifugation, PRP was transferred into an empty 50mL falcon with 1 μ M of PGI₂ and centrifuged at 1000g for 10 minutes at room temperature. The supernatant was discarded leaving the platelet pellet. Platelets were re-suspended in 3mL and 25mL of warmed ACD and Tyrode's buffer (NaCl 134 μ M, KCl 2.9 μ M, Na₂HPO₄·12H₂O 0.34 μ M, NaHCO₃ 12 μ M, HEPES 20 μ M, MgCl₂ 0.998 μ M) respectively with 1 μ M of PGI₂, gently mixed by inverting the tube 6 times and centrifuged at 1000g for 10 minutes at room temperature. Finally, the supernatant was removed, and washed platelets were re-suspended in either Tyrode's or M199 serum free media (ThermoFisher, #31150022, Massachusetts, USA). Washed platelets were used for preplatelet/barbell kinetic studies, TRAP degranulation and tubulin immunofluorescence.

2.4.4 Haematology Analysers

All human blood samples were acquired on the XN1000 haematology analyser (Sysmex, Kobe, Japan) where a full blood count was generated. The XN1000 is a high

throughput instrument which requires 88 μ L of whole blood to quantify multiple clinical parameters including leukocyte, erythrocyte and platelets parameters. Platelet parameters include counts (using fluorescence, (PLT-F); optical, (PLT-O); and impedance (PLT-I) methods), IPF% and immature platelet count (IPF#), platelet crit (PCT), platelet distribution width (PDW), MPV, lower and upper platelet discriminators (PL and PU) and platelet large cell ratio (P-LCR).

Platelet counts were determined by fluorescence in the PLT-F channel. PLT-F is calculated from cell size (FSC) and side scattered fluorescence intensity (SFL) of a novel propriety dye, Fluorocell-PLT®. Fluorocell-PLT® specifically binds to platelets allowing their discrimination from both RBC and leukocytes.

IPF is also measured in the PLT-F channel. Using systematic algorithms, IPF is determined by the fraction of platelets with the highest FSC and Fluorocell-PLT® SFL intensity. Internal quality control material (XN check, Sysmex, Kobe, Japan) was tested daily to ensure instrument performance throughout the study. The instrument was also enrolled into a national external quality assurance scheme (UKNEQAS, Watford, UK). To determine normal ranges for each cellular parameter, blood samples from 40 healthy volunteers (control cohort) were analysed and normal ranges are represented as the mean value $\pm$ 2 standard deviations.

For mice, the platelet count from anticoagulated whole blood was determined by the Pentra ABX 60 (Horiba, Montpellier, France). The Pentra ABX utilises a double hydrodynamic sequential system which quantifies platelet counts and MPV using electrical impedance. Quality control blood samples are acquired daily on the Pentra

ABX 60 analysers and results are archived to monitor sample analysis and system stability.

2.5 Blood Films

Blood films were performed using room temperature or 37°C whole blood. 7µL of blood anticoagulated in EDTA, citrate or hirudin was applied to the uppermost central region of a microscope glass slide (H76 x W26 x D1mm; FisherScientific, #13192131, Massachusetts, USA). A second clean slide was placed above the droplet of blood at a 45° angle. As the blood spread laterally along the second slide, it was dragged steadily down the face of first slide creating a smear of blood. Blood films were monitored whilst air drying and once dry, stained immediately using REASTAIN® modified Giemsa Quik-Diff Kit (Reagen, #102164, Toivala, Finland). Using this kit, blood films were fixed in a methanol reagent for 10 seconds and stained with Eosin Y red solution for 10 seconds and methylene blue solution for 7 seconds. Between staining steps, excessive reagents were washed with deionised water (ddH₂O) and slides were carefully dabbed with tissue tapping the slide in a vertical position and left to air dry. Once air dried, blood films were mounted in DPX mounting media (Sigma, #06522) and imaged on the DM6000 Leica widefield microscope at x60 magnification. Platelet cytoplasm was stained violet and granules dark purple (Figure 2.1).

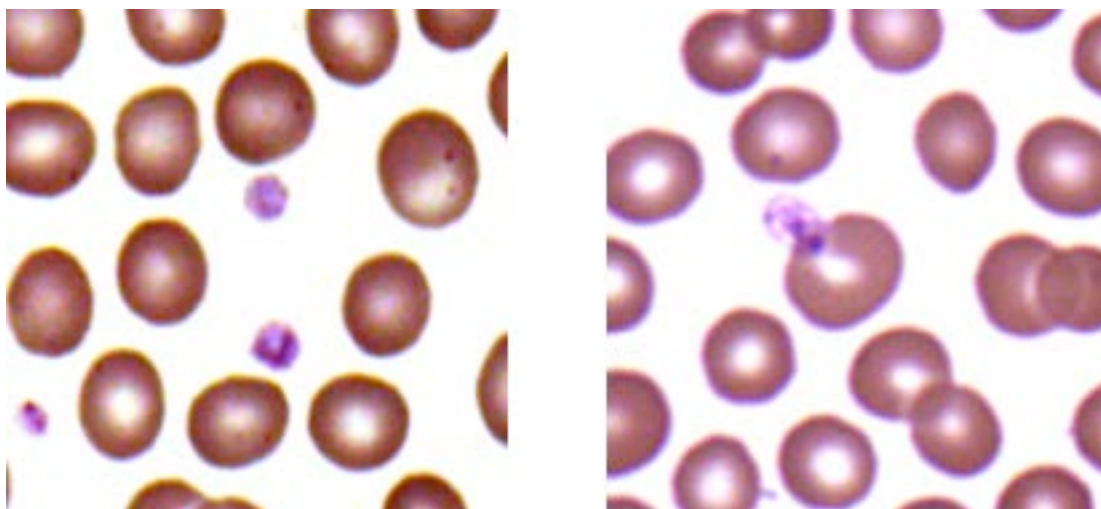


Figure 2.1: REASTAIN® Modified Giemsa Quik-Diff Labelling of Blood Films

Blood films were fixed in methanol and stained with Eosin Y and methylene blue. Platelets are easily identified by their violet cytoplasm and dark purple, platelet granules. Larger RBC are stained red/brown with their classical bioconcave structure.

2.6 Drug and Cytokine Treatments

Whole blood or washed platelets were treated with various drugs to determine the effect on preplatelet/barbell kinetics. Table 2.1 lists details regarding drug type, concentration and function. details regarding protocols can be found in respective chapter methodologies.

Table 2.1: Drug and Cytokine Treatments.

Treatment	Function
Eptifibatide	$\alpha_{IIb}\beta_3$ Inhibitor
Nocodazole	Microtubule Destabilising Reagent
Cytochalasin D	Actin Depolymerising Reagent
Thrombopoietin	Growth Hormone

2.7 Antibodies, Probes and Dyes

An extensive list of antibodies, probes and dyes used for human and mouse studies are displayed in Table 2.3 and 2.4 respectively. Antibodies were titrated to determine the minimal saturating concentration to get best signal to noise ratio whilst maintaining good image quality when performing ImageStream^X acquisition.

Labelling protocols are included within respective chapters including details regarding isotype controls and gating strategies.

Table 2.2: Antibodies, Dyes and Probes used for Human Experiments

Antibody or Probe	Clone	Isotype	Company / Ref	Dilution: Blood	Dilution: Washed Platelets
CD62p (BV421)	AK4	Mouse IgG1	BioLegend #304926	1/100	1/100
CD42b (BV421)	HIP1	Mouse IgG1	BioLegend #303930	1/300	N/A
CD61 (FITC)	RUU- PL7F12	Mouse IgG1	BD #348093	1/25	1/25
CD62p (PE)	VI P-44	Mouse IgG1	BioLegend #304906	1/100	N/A
CD61 (PE)	VI PL2	Mouse IgG1	BD #555754	N/A	1/50
CD42b (PE)	HIP1	Mouse IgG1	BD #555473	1/50	1/50
HLA I (APC)	W6/32	Mouse IgG2a	BioLegend #311410	1/50	N/A

SiR Tubulin (AF 674nm)	N/A	FMO	Spirochrome #CY-SC002	1/250	1/2500
TO (AF 512nm)	N/A	FMO	Sigma #390062	1/5000	N/A
Mitotracker CMXRos (AF 599nm)	N/A	FMO	ThermoFisher #M7512	1/500	1/2000

AF: Alexa Fluor; TO: Thiazole Orange; FMO: Fluorescence Minus One

Table 2.3: Antibodies, Dyes and Probes used for Mouse Experiments

Antibody or Probe	Clone	Isotype	Company / Ref	Dilution: Blood
CD62p (BV421)	VI P-44	Mouse IgG1	BioLegend #304926	1/100
CD41a (FITC)	MWReg30	Rat IgG1	BioLegend #133904	1/100
CD41a (APC)	MWReg30	Rat IgG1	ThermoFisher #17-0411-82	1/100
Streptavidin (FITC)	N/A	FMO	BioLegend #405201	1/10
TO (AF 512nm)	N/A	FMO	Sigma #390062	1/5000
SiR Tubulin (AF 674nm)	N/A	FMO	Spirochrome #CY-SC002	1/250

AF: Alexa Fluor; TO: Thiazole Orange; FMO: Fluorescence Minus One

2.8 Imaging Flow Cytometry

The ImageStream^x (Figure 2.2a) combines population statistics of CFC and the spatial depiction afforded by basic microscopy. This instrument performs high-resolution multispectral imaging using either brightfield, SSC and/or fluorescence. Objective lenses consist of either 20, 40 or x60 magnification which produce a pixel area of 0.1, 0.25 and 1 μm^2 respectively with a maximum field of view width of 128 μm . To conduct multispectral imaging, hydrodynamically focussed cells are trans-illuminated by brightfield (BF) and orthogonal lasers. Emitted light is collected by a high-resolution objective lens and relayed to a dichroic filter stack. Light from the stack is organised into spectral bands and projected on a CCD camera (Figure 2.2b). The CCD camera operates in a time delayed integration (TDI) mode and tracks cells passing through a band of pixels (total of 256 rows) in synchrony with cell velocity, to achieve a high level of fluorescence sensitivity without streaking. The ImageStream^x 2 camera system has the potential to acquire a maximum of 12 images per cell - 2 BF, 1 side scatter (SSC) and 9 fluorescent parameters - and process up to 5000 events per sec, ensuing high throughput acquisitions to overcome the subjective analysis of microscopy. For 1 camera systems (used throughout this thesis), BF imaging can be performed in channel (CH) 01, CH04 or CH06. SSC is imaged in CH06 only. Table 2.5 shows a list of excitation lasers and potential fluorochromes used by the ImageStream^x single camera system. Fluorochromes, dye and probes used in this thesis are highlighted in black.

A



B

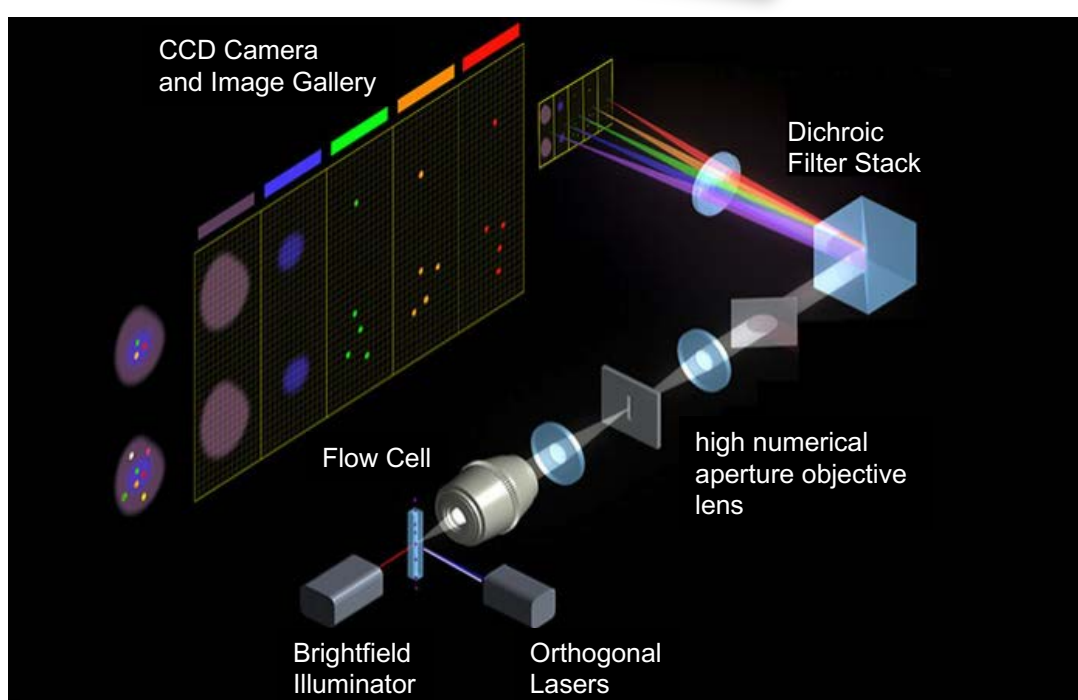


Figure 2.2: ImageStream^X MKII Amnis® by Luminex Corp

(A) ImageStream^X MKII instrument. (B) Multispectral imaging by ImageStream^X. Cellular events are hydrodynamically focussed through the flow cell where they are trans-illuminated by brightfield and orthogonal lasers. Light emission is collected by a high numerical aperture objective lens where it is transmitted into a dichroic filter stack. Inside this element, light sources are sorted into spectral bands and therefore different angles and transmitted at varied locations on the CCD camera. These locations represent sub-images of single cell dependent on the fluorochrome and imaging technique (such as brightfield or SSC) used. Images were taken from the Luminex Corp Imaging flow cytometry website. See link below.

<https://www.luminexcorp.com/wp-content/uploads/2018/11/Screen-Shot-2018-11-29-at-12.13.43-PM.png>

Table 2.4: ImageStream^X Fluorochrome Chart for a Single Camera System

Channel	Band	Excitation Laser (nm)		
		405	488	642
1	435-505	DAPI, BV421, Hoechst, PacBlue, CascadeBlue, eFluor450, DyLight405, CFP, LIVE/DEAD Violet		
2	505-560	BV510, PacOrange, Cascade Yellow, AF430, eFluor525, QD525	FITC, AF488, GFP, YFP, DYLight488, PKH67, Syto13, LysoTrackerGreen, MitotrackerGreen, Thiazole Orange, CellTrace™ CFSE	
3	560-595	QD565, QD585, eFluor565	PE, PKH26, DSRed, mOrange, Sytox Orange, Cy3, CellTrace™ Yellow	
4	595-642	QD625, eFluor625, BV605	PE-TexRed, PI, RFP, QD625, eFluor625, MitotrackerCMXRos	
5	642-745	QD705, eFluor700, BV711	PE-Cy5, PE-AF647, 7AAD, PerCP, PerCP-Cy5.5, DRAQ5, QD705	APC, AF647-680, Cy5, DyLight649, PE-AF647, PE-Cy5, DRAQ5, PerCP, PerCP-Cy5.5, SiR Tubulin, CellTrace™ Red
6	745-780	QD800, BV786	PE-Cy7, PE-AF750, QD800	APC-Cy7, APC-AF750, APC-H7, Cy7, AF750, PE-Cy7, PE-AF750

2.8.1 Image Data Exploration and Analysis Software (IDEAS)

With a robust image analysis platform, it is possible to discriminate between different shapes of cells that appear to be immunologically identical based on a comparison of complex morphological parameters. In summary, IDEAS uses masks and features to discriminate cells of interest. Morphology is determined in all fluorescent channels and BF. From this, spatial distribution of fluorophores, on a single cell, is defined in context with BF imaging. Alternatively, systematically designed masks and features are used to define and quantify characteristics of a single cell, based on cellular compartments and object-based measurements respectively.

Masks can be created based on BF, any fluorescent channel and SSC light. There are three groups of masks: default, custom and combined. The default BF mask is generally used to gate focused cells and (if necessary) exclude coincidence events based on BF cell area over aspect ratio. Default fluorescent masks are also useful for gating a population of interest. However, to determine the morphology or spatial distribution of fluorescent signals on a cell-by-cell basis, masks must be custom designed and/or combined with features using Boolean logic.

Upon acquisition, ImageStream^X generates raw image files (rif) which were uploaded in IDEAS and synced to compensation matrices to create compensated image files (cifs) and a data analysis files (dafs). The rifs consist of image content, pixel intensity data (count and location), instrument settings and calibration values attained during the acquisition. Pixel by pixel, compensation is performed during the creation of a cif. During this process, the IDEAS uses the compensation matrix values to correct the imagery by removing artefacts, background and gain; aligning images and cropping coincidence events into individual images to create a picture gallery. Like CFC, the

compensation matrix removes or maximally reduces the degree of fluorescence spill over from one channel to another. Compensation matrices were developed using single stain fluorescent controls. Single stain controls were acquired in all channels, with all lasers on (405, 488 & 642nm) and the SSC and BF imaging switched off. An example of a compensation matrix developed using ImageStream^X can be found in appendix 2.1.

Once compensated, image analysis was performed in the daf, from this file, features, populations, graphs and statistics were defined; features were measured, and images optimised. Image analysis templates were linked to dafs, saved as templates and applied to other dafs to compare differences between cohorts and/or experimental conditions.

2.8.2 Image Optimisation

Display properties improve the quality of the pixel content by adjusting the mapping of the pixel intensities from each acquired image channel. This does not alter the pixel values measured during acquisition but will improve image quality by setting an average pixel range to exclude saturated dim images. RMS- (Root Mean Squared-) gradient measures surface roughness. Blurred objects tend to be smooth as detail is lacking. The rougher the surface of an object the more focussed and therefore the greater the RMS-gradient value. For this study, focussed fluorescent images were determined by an RMS-gradient >20 and BF by >50.

Care must be taken when excluding unfocussed images during acquisition as this may give an inaccurate representation of your total cell population (this is particularly important for quantification, ratios within whole blood and detecting defective cell

morphology). For example, ImageStream^x is commonly used to identify and quantify abnormal RBC in sickle cell disease in humans [393]. If unfocussed images are rejected during acquisition, no sickle blood cell images would be detected due to the twisted and unfocussed morphology. In contrast, unfocussed images will artefactually increase image morphometric measurements. In this case only focussed images are analysed.

2.8.3 Truth Populations

Truth populations depict the cells of interest which represent morphological phenotypic differences. From the optimised images, a population of interest was gated, and individual images were selected which best represent the target truth populations. By selecting two or more morphologically different populations it provides the information necessary for IDEAS to discriminate between such events. For example, for barbell platelets discrimination it was essential to select a truth population consisting of barbells and another of single platelets (explained in detail in Chapter 3). The channel in which the truth populations are developed is important as it must be kept consistent when customising a mask.

2.8.4 Pixel Masks

Morphology masks identify a set of pixels that are within a region of interest of the designated truth populations. The specific region of the image depicted by the mask is used for feature-value calculations. There are 15 custom morphology mask functions available: dilate, erode, fill, inspire, intensity, interface, morphology, object, peak, range, skeleton, spot, system, threshold and valley (see Table 2.6 for list and

description). Masks can be combined to include two functions or to exclude one of the two. The latter technique is a useful tool for measuring cell membrane or cytoplasm characteristics only.

Pixel masks used for preplatelet and barbell platelet analyses were morphology, erode and skeleton thin.

Table 2.5: Pixel Mask: Type and Function

Masks	Function
Adaptive Erode	Identifies pixels which form a circle that touch the input boundary
Component	Input mask with multiple sections which can be ranked
Dilate	Adds a selected number of pixels to the edge of an image
Erode	Removes a selected number of pixels from the edge of an image
Fill	Fills holes in starting mask
Inspire	Includes all pixels above background values
Intensity	Includes pixels within the upper and lower raw intensities
Interface	Measures the contact point of two objects within a single image
Morphology	Includes all pixels within the outermost image contour
object	Identifies the area which closely resembles that of the image
Peak	Identifies local areas of maxima intensities
Range	Selects components of an image based on intensity values
Skeleton Thick	Barebone structure of an image based on pixel intensities
Skeleton Thin	1-pixel wide skeletal line of an image
Spot	Identifies bright or dark regions of an image
System	Segments images based on pixel clustering
Threshold	Excludes pixels outside a defined range
Valley	Rectangular mask which sits between two regions of intensity

2.8.5 Features

A feature is a mathematical model that contains quantitative and positional information regarding an image. It is applied to specific locations of an image determined by a mask which identifies a region of interest based on pixel content. Features are based on size, shape, location, texture, signal strength, comparison and system measurements (Table 2.7). These feature categories are defined by the IDEAS application and are used to create features for user image measurements. Feature design relies on pre-development of a morphology mask and truth populations. Features generated from such parameters can be listed dependent on median relative difference (RD) scores. The features with the highest median RD are theoretically the best discriminators of the selected truth populations synced to a specific mask.

Features used for preplatelet and barbell platelet analyses include RMS-gradient, compactness, symmetry2 combined with height, aspect ratio, minor axis intensity and area of a morphology mask; major axis intensity of a skeleton thin mask; diameter of an erode mask.

Table 2.6: Feature Categories and Function

Feature Category	Measurement
Size Features	
Area	Size of the mask in μm^2
Diameter	Diameter of the mask based on area
Height	Longest dimension of a bounding rectangle of a mask
Length	Longest dimension of a mask
Major Axis	Longest dimension of an ellipse of best fit
Minor Axis	Shortest dimension of an ellipse of best fit
Major Axis Intensity	Longest dimension of intensity weighted-ellipse of best-fit
Minor Axis Intensity	Shortest dimension of intensity weighted-ellipse of best-fit
Perimeter	Boundary length of a mask in μm
Shape Features	
Aspect Ratio	The minor axis divided by the major axis
Circularity	The degree of deviation of the mask from a circle
Compactness	The degree of packing of the pixel intensities within a mask
Elongatedness	Ratio of height over width of the bounding box
Lobe Count	Number of lobes of the event based on weighted symmetry
Symmetry 2	The tendency of an image to have a two-fold axis of symmetry
Texture Features	
Gradient RMS	The roughness of an image to determine the level of focus
Other	
Localisation	Measurements to determine position of fluorescent intensity
Comparison	Measurements to determine differences in fluorescent intensity
System	Measurements to determine system wide metrics

2.9 Flow cytometry

All flow cytometry assays were performed using the BD Accuri™ C6. The instrument was quality checked prior to use with BD™ Cytometer Setup and Tracking (CS&T)

beads (BD, #911013) to ensure daily quality control and performance criteria. Compensation was not required as selected markers throughout this thesis were conjugated to FITC (fluorescein isothiocyanate) and APC (Allophycocyanin; two fluorochromes which contain emission spectrums which do not overlap)

For all flow cytometry assays, human or mouse whole blood (5 μ L) was labelled with 45 μ L of PBS containing a platelet specific marker (e.g. FITC CD41a or APC CD41a) for 15 minutes at room temperature in the dark and fixed in 50 μ L of 4% formalin (details regarding labelling protocols etc can be found in the respective assay methodologies). Prior to acquisition, all samples were diluted in PBS to a final concentration of 1/1000. As platelets cannot be directly discriminated from RBC using Log FSC-H and SSC-H measurements, they were discriminated by plotting Log fluorescence of a platelet specific marker (e.g. APC CD41a) and Log FSC-H (Figure 2.3).

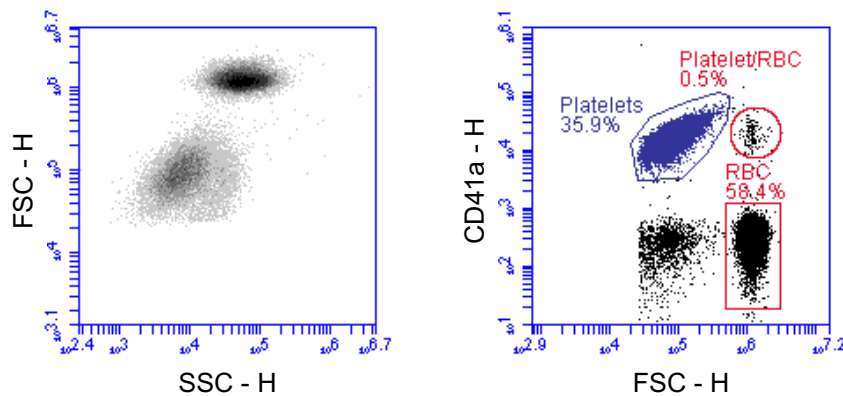


Figure 2.3: Identifying Platelets by Flow Cytometry

Left scatter plot shows all whole blood cellular events depicted by Log FSC-H and SSC-H. Right scatterplot shows using a platelet specific marker (APC CD41a). The platelet cluster (represented by blue events) can be discriminated from RBC (red square) and platelet/RBC co-incidence events (red circle). H: height. RBC: red blood cell.

2.10 Reticulated Platelet Assay

RP are newly formed platelets described as being greater in size and containing a high RNA content compared to terminally differentiated (mature) platelets. Therefore, it is possible to quantify RP using a two-colour flow cytometry assay based on FSC light (size) and fluorescence intensity of nucleic acid dyes such as TO (1-methyl-4[(3-methyl-2(3H)-benzothiazolylidene) methyl]-quinolinium 4-methyl benzene sulfonate). BD Retic-Count™ (BD, #349204) is a specialised reticulocyte reagent which contains 0.01mg/mL TO in PBS with 0.1% sodium azide and is commonly used to measure RP by flow cytometry. BD Retic-Count™ binds to platelet RNA and is excited by the 488nm (FL1) laser emitting a fluorescent spectral band at 530nm. With the addition of a platelet specific marker RP are quantified.

RP were measured in mice. For each sample, 5µL of whole blood were labelled with 45µL of filtered PBS containing APC, anti-mouse CD41a (α_{IIb} , 1/100: ThermoFisher, #17-0411-82) in a sterile 5mL round bottom Falcon tube and incubated for 15 minutes in the dark at room temperature. To each CD41a labelled sample tube, cells were resuspended in 950µL of BD Retic-count (containing 0.01mg/mL TO) and incubated for 30 minutes. Samples were fixed with 4% (final concentration 2%) filtered formaldehyde for 10 minutes at room temperature and resuspended in a total of 5mL PBS, giving a final dilution of 1:1000. Reticulated platelets were quantified on the BD Accuri™ C6.

An APC Rat IgG1 κ isotype control (1/100: ThermoFisher, #17-4301-82) was used to generate the CD41a positive gate and TO fluorescence minus one (FMO) with 0.1% sodium azide in PBS (S2002 Sigma) was used to determine the TO positive gate. RP were determined by CD41/TO expression (Figure 2.4).

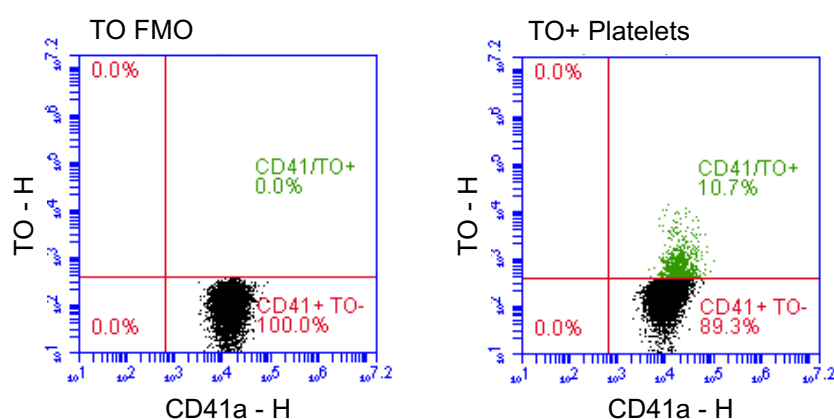


Figure 2.4: Determination of Reticulated Platelets

Platelets were doubly labelled with a platelet specific marker (e.g. APC CD41a) and 0.01mg/mL thiazole orange (TO). To determine the TO positive signal, platelets were labelled with APC CD41a only (TO FMO). CD41a/TO positive platelets are displayed by green whereas, black signifies CD41a positive/TO negative platelets. Data shown is representative of a single experiment. TO was detected in FL1; CD41a in FL4. FMO: fluorescence minus one; H: height.

2.11 TRAP Degranulation Assay

Thrombin activates PAR receptors by cleaving the extracellular N-terminus to expose an agonist peptide ligand (SFLLRN) resulting in degranulation [419]. TRAP (Thrombin Receptor Activating Peptide (TRAP; Sigma, #T1573) is a synthetic peptide containing SFLLRN which directly activates PAR without cleavage.

Washed platelets ($100 \times 10^6/\text{mL}$) were labelled with the cytosolic dye CellTrace™ yellow ($2\mu\text{M}$; emission 579nm; ThermoFisher, #C34573) for 30 minutes at room temperature in the dark. Labelled platelets were centrifuged at 1000g for 10 minutes with $1\mu\text{M}$ PGI₂ to remove dye precipitates and resuspended in Tyrode's buffer ($100 \times 10^6/\text{mL}$). Once dye precipitates were discarded, CellTrace™ yellow labelled platelets were exposed to increasing concentrations of TRAP (1, 10, 100 or 1000 μM) for 30 minutes at room temperature to induce degranulation. Following TRAP exposure, platelets were fixed in 2% formalin for 10 minutes, centrifuged at 1000g to

remove leaked dye. Platelets were identified by ImageStream^X using anti-human FITC CD61 (1/25, BD, #348093) and granule release was determined by anti-human BV421 CD62p (1/100, BioLegend, #304926). For negative controls, washed platelets were incubated for 30 minutes without TRAP and a BV421 mouse IgG1 k isotype control (1/100, Clone: MOPC-21, BioLegend, #400158) was used to set a positive gate at 1% of the negative peak (Figure 2.5).

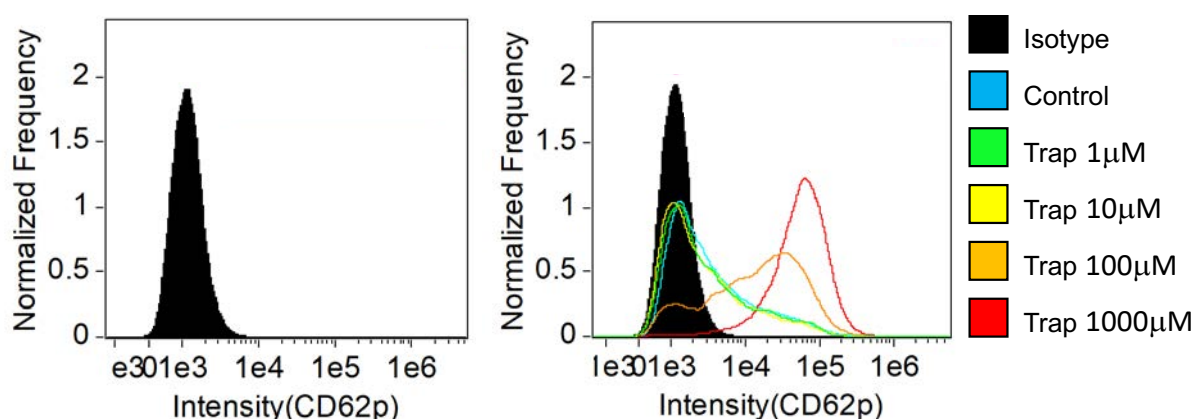


Figure 2.5: Determination of TRAP-Induce P-Selectin (CD62p) Exposure

A BV421 mouse IgG1 k isotype control (black peak) was used to determine P-selectin (CD62p) positive events. The histogram on the right is an overlay of CD62p expression in platelets in response to increasing concentrations of TRAP as shown on the right. The data is representative of a single experiment.

2.12 Immunofluorescence and Microscopy

Glass, 13mm coverslips (VWR, #631-1578, Pennsylvania, USA) with a thickness of 0.16 - 0.19mm (Structured Illumination Microscopy (SIM) requires a coverslip thickness >0.15 as it uses refractive index to construct images) were pre-incubated with 500μL of poly-L-lysine (0.01%; Sigma, A-005-C) in a 24-well flat bottom plate (StarLab, #CC7672-7524, Milton Keynes, UK) for at least 1h. A concentration of 5×10^6 /mL

platelets were used for all immunofluorescence labelling. Unless otherwise stated, all staining steps were carried out at room temperature in non-sterile conditions.

PRP was prepared from human citrate whole blood under strict 37°C temperature control, fixed in an equal volume of prewarmed 4% formalin in PBS (2% final concentration), centrifuged at 200g for 10 minutes onto poly-L-lysine coated 13mm coverslips and washed 3 times in 1mL of PBS. Platelets were permeabilized in 1/1000 triton X in PBS (Sigma, #T8787) for 10 minutes, washed 3 times in 1mL PBS and non-specific binding was blocked with 1% goat serum (blocking buffer; Sigma, G9023) and filtered 2% BSA (bovine serum albumin; Sigma, #A9647) in PBS for 1h. The blocking buffer was aspirated, and tubulin was labelled with an anti-mouse α -tubulin monoclonal antibody (1/500; Sigma, #T8203) in blocking buffer for 1h to avoid species cross adsorption, washed 3 times in 1mL PBS and secondary labelled with goat anti-mouse IgG (H+L) Alexa Fluor 488 (1/1000; Invitrogen, #A-11001, California, USA) for 30 minutes. Following secondary labelling, platelets were washed a final 3 times with 1mL PBS and coverslips were air dried and mounted (on a microscope glass slide; H76 x W26 x D1mm; FisherScientific, #13192131) in 10 μ L of vectashield vibrance aqueous solution (Vector Laboratories, #H-1700, California, USA) and α -tubulin platelets were imaged at x60 magnification using Leica DM6000 and x100 for super-high resolution SIM microscopy. Both microscopes utilise widefield-based imaging techniques.

Super-high resolution SIM microscopy utilises laser-based widefield microscopy with a movable diffraction grate. As emitted light passes through, it creates stripes of fluorescence (forming a Moiré effect). When acquiring a single image, several raw images are collected through the diffraction grate at multiple angles and reconstructed to generate a final super-resolved image.

Although the above tubulin labelling method is an established protocol used to define a distinct cytoskeletal platelet margin band, background signal was determined using a negative control which entailed labelling as above but without the anti-mouse α -tubulin step.

2.13 Platelet Aggregometry Test

Eptifibatide (Sigma, #SML1042; commercially known as Integrilin®, Millennium Pharmaceuticals, Essex) is a synthetic cyclic heptapeptide which originates from a disintegrin protein (P22827) associated with the venom of the south-eastern pigmy rattle snake *Sistrurus miliarius barbouri*). This antiplatelet drug binds to the RGD recognition site of $\alpha_{IIb}\beta_3$ causing a conformational change preventing aggregation.

The PAP-8E platelet aggregometer (Bio/Data Corporation) was used to assess the inhibitory effects of eptifibatide. The PAP-8E uses an ultraviolet (UV) 430nm light source to measure light transmission. Activation chambers and tubes were pre-heated to 37°C. PPP was used to set 100% light transmission. 225 μ L of PRP generated from human citrate whole blood was transferred into aggregometry tubes in each test chamber with a micro-stirrer bar. Samples were stirred at 1200rpm and a 0% baseline established before addition of 25 μ L of ADP (working concentration of 10 μ M) with or without eptifibatide (working concentration of 10 μ M). Platelet aggregation was recorded for 8 minutes

2.14 In vivo Biotinylation of Platelets

In vivo biotinylation, labels all circulating cells including platelets, RBC and white blood cells (WBC) for cell tracking and survival/lifespan quantification. NHS(N-

HydroxySuccinimido)-Biotin ($C_{14}H_{18}O_5N_3S$) ester biotin injected intravenously, covalently binds to proteins and peptides situated on platelets with little effect on normal function. This process is rapid and stable. Biotin positive platelets can be detected using a two-colour flow cytometry approach using a platelet specific marker (e.g. CD61 or CD41a) and a streptavidin conjugated antibody. Immediately after biotin injection, all target cells (i.e. platelets) become positive for biotin. After 24 hours, this allows the resolution of biotin negative newly released immature platelets from biotin positive mature platelets. The loss of biotin labelling over days can also be used to estimate platelet lifespan.

To maintain sterility NHS-biotin was prepared for injection in a tissue culture hood. NHS-biotin (Sigma, H1759) was resuspended in DMSO to 40mg/mL using a vortex and stored in -20°C. Prior to use, NHS-biotin was thawed at room temperature and further diluted in saline to 4mg/mL using a vortex to ensure no precipitates were present. WT C57/6 mice (n=4) were injected intravenously twice (30 minutes apart) with 150 μ L of NHS-biotin (4mg/mL) in saline into the lateral tail artery to ensure that 100% of platelets were labelled. Biotin positive platelets were measured in murine whole blood by flow cytometry 1h (Day 0) and 24h (Day 1) after the 2nd intravenous NHS-biotin injection. Blood samples were taken from the tail vein on Day 0 and by terminal bleed on Day 1.

To determine NHS-biotin positive platelets in WT mice, 5 μ L of citrate anticoagulated whole blood was labelled with anti-mouse CD41a-APC (1/100, ThermoFisher, #17-0411-82) and FITC-conjugated streptavidin (1/10, BioLegend, #405201, California, USA) for 30 minutes in the dark at room temperature. FITC-streptavidin FMO and non-injected control mouse blood was used as negative controls to determine platelet

biotinylation (Figure 2.7). An APC rat IgG1 k isotype control (1/100, ThermoFisher, 17-4301-82) was also used to generate APC CD41a fluorescence threshold.

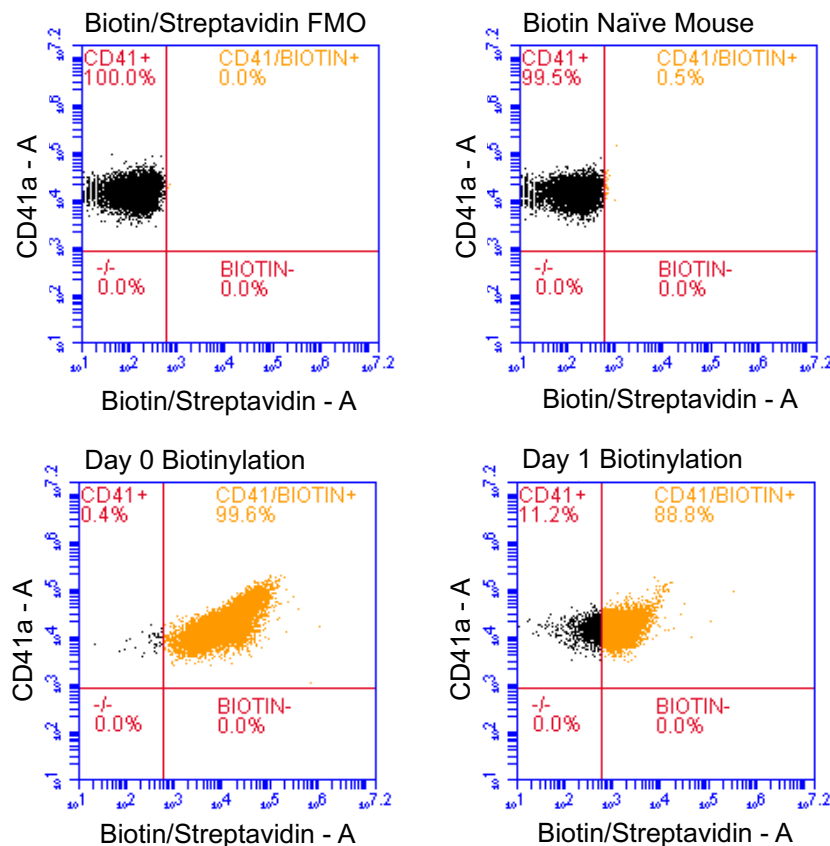


Figure 2.6: Measurement of In Vivo Biotinylated Platelets

Biotinylation was measured using FITC conjugated streptavidin protein. To determine the platelet biotin positive gate, biotinylated mouse blood was labelled with CD41a only (top left; Biotin/Streptavidin FMO) and a double labelled (CD41a/streptavidin) unbiotinylated mouse (top right). Platelet biotinylation was measured 30 minutes following NHS-biotin injection (Day 0) to confirm ~100% platelets were biotinylated (orange events; CD41a/Biotin) and 24 hours later to show new immature biotin negative platelets (black events determined by CD41a positive / biotin negative). Data is representative of a single experiment. FMO: fluorescence minus one.

Newly formed immature platelets on Day 1 were determined as CD41a positive/streptavidin negative (black; lower right scatter plot). As antibodies conjugated to FITC and APC were used in this experiment, no compensation was required.

2.15 GPIb α -mediated Platelet Depletion Assay

Prior to platelet depletion, mice were bled by tail bleed into a 1.5mL Eppendorf containing 20 μ L of 4% sodium citrate for all baseline measurements. C57B/6 WT mice (n=10) were pre-weighed and injected into the peritoneal (ip) with 150 μ L of sterile PBS containing a polyclonal rat anti-mouse GPIb α antibody (final concentration of 1.5 μ g/g of body weight; Emfret, Ref: R300, EibelStadt, Germany) to induce platelet depletion. To determine changes in platelet counts, additional tail bleeds were taken on days 1, 5 and 8 following platelet depletion (n=5). For preplatelet and barbell analyses, mice (n=5) were bled via a terminal bleed.

2.16 Data Handling and Statistical Analysis

A summary of the data analysis strategy used is intended here. Details regarding tests and significance are highlighted in respective figures throughout the thesis. All data analysis was carried out using GraphPad Prism v8.4.3 (GraphPad Software, California, USA). As the sample size of all data sets was <50, Shapiro-Wilk normality tests were employed to determine normality. For two dependent parametric or non-parametric data sets, a paired t test or a Wilcoxon Signed Rank test was performed. In contrast, for two independent parametric or non-parametric data sets, an unpaired t test or a Mann-Whitney U test were performed.

For parametric independent data sets consisting of >2 data sets a one-way ANOVA was used. In combination with one way ANOVA, a multiple comparison Tukey test was used to compare differences between all mean values and Dunnett's test to determine differences in relation to the control. In contrast, a two-way ANOVA was used to determine differences between two independent variables in relation to time. To

determine individual significant differences at each time point, Bonferroni or Sidak multiple comparison tests were used.

To determine the relationship between two continuous variables, a Pearson's Correlation Coefficient (r) was used.

A p value of < 0.05 (*) was considered significant. Data are presented as ± 1 SD of the mean.

**Chapter 3 - ImageStream^x and IDEAS:
Methodological Development and
Validation of Discriminating
Preplatelet Maturation Events**

3.1 Introduction

In this methodology chapter, we describe and discuss the development, validation and limitations of discriminating preplatelets and barbell platelets directly in whole blood. As mentioned in detail in the “Introduction” and “General Methods” sections, preplatelets are thought to lack immunological markers for their discrimination, rendering techniques such as flow cytometry incapable for their detection and subsequent quantification. Considering this, there is a need to design high throughput image-based methods which have the power to discriminate preplatelet and barbell platelets in whole blood.

IFC combines CFC with microscopy achieving high throughput morphological and spatial measurements at a single population and/or cellular level (see introduction for full description of IFC and the benefits of using such methods in research). The ImageStream^x is an example of next generation IFC (see sections 2.6 “Introduction” & 2.8 “General Methods” for instrument details, specifications and utilisation). Like flow cytometry, this instrument applies hydrodynamic focussing to ensure a single cell is imaged at any one time.

The ImageStream^x uses a robust image analysis platform IDEAS that generates pixel masks and features to score and discriminate cells of interest based on quantitative morphological metrics such as size, shape, texture and intensity [396]. Such high throughput morphological quantification in combination with hydrodynamic focussing means it should be possible to discriminate preplatelets and barbell platelets directly in whole blood with ImageStream^x using various morphological criteria.

The focus of this chapter is to utilise the power of ImageStream^x and accurately discriminate preplatelets and barbell platelets in whole blood. This methodology will be

used throughout the thesis to understand the physiological relevance of preplatelet maturation events in normal and acquired thrombocytopenic blood (Chapters 4, 5 & 6). These findings will emphasise the importance of developing new high throughput image-based methods to improve current immature RP measurements which could also be implemented into a clinical setting to improve diagnosis and management of thrombocytopenia.

3.2 Summary of Methods

25 μ L of whole blood was added to 25 μ L of PBS containing anti-human BV421 CD62p (1/100, #304926, BioLegend), anti-human FITC CD61 (1/25, #348093, BD) and anti-human PE CD42b (1/50, #555473, BD) and incubated for 15 minutes at 21 or 37°C in the dark and snap fixed with 50 μ L of 4% formalin diluted in PBS (0.22 μ m filtered; final formalin concentration of 2%). Alternatively, 25 μ L of mouse whole blood was labelled with 25 μ L of PBS containing anti-mouse BV421 CD62p (1/100, #304926, BioLegend) and anti-mouse FITC CD41a (1/100, #133904, BioLegend).

The threshold for CD62p positive signals for human and mouse platelets was determined using BV421 mouse IgG1 κ (1/100, MOPC-21, #400158, BioLegend) and BV421 rat IgG1 λ (1/100, #562604, BD) isotype controls respectively. Compensation matrices were developed from single stain controls. For each of these controls, 1000 positive images were acquired in all fluorescent channels with BF and SSC switched off. Compensation matrices were generated in IDEAS using the compensation wizard (see Appendix Table 2.1 for an example of a compensation matrix generated by IDEAS).

Human and mouse platelet analyses were performed using the specific platelet identifiers FITC CD61 or PE CD42b (human) or FITC CD41a (mouse) fluorescence respectively. For each sample, 10,000 positive platelet images were acquired. Focussed images of fluorescently labelled platelets were determined by Gradient RMS >20 (see section 2.8.2 “Image Optimisation”). Focussed platelet images were initially interrogated manually with the aid of aspect ratio and area of BF images to identify platelets, preplatelets and barbell-shaped platelet structures in whole blood. Following this 10,000 FITC CD61/PE CD42b positive human platelet images were acquired (10,000 FITC CD41a images for mice). Preplatelets and platelets were determined by circularity and diameter. Whereas, in order to discriminate barbell-shaped structures from the platelet cloud, truth populations consisting of single circular platelets (“singlets”), barbell platelets containing a thin cytoplasmic bridge (“barbells”; see images in Appendix Figure 4.2) and barbell-shaped microaggregates consisting of two distal platelet structures (“microaggregates”; see images in Appendix Figure 3.2). 100 images were selected for each truth population. These truth populations were discriminated manually by generating median RD statistical reports. For manual analyses, morphology and skeleton thin pixel masks were designed and in combination with the truth populations, features were designed and ranked using a median RD report (the higher the RD score the greater the discriminating potential of the feature). Separate RD reports were generated based on singlets vs barbells and singlets vs microaggregates. From these reports, appropriate features were used to discriminate the truth populations (described in detail below).

3.3 ImageStream^X IDEAS Methodologies

3.3.1 ImageStream^X-MKII Whole Blood Acquisition

The majority of ImageStream^X optimisation was performed in human blood and then recapitulated in mice. To determine platelet morphology and ultimately preplatelet maturation events (preplatelets and barbell platelets) in anticoagulated blood samples, all antibody-labelled blood samples were diluted 1/80 with PBS, fixed in 2% formalin and acquired using the ImageStream^X. Preplatelet and barbell analyses were performed using citrate blood incubated for 15 minutes at 37°C (the effect of anticoagulation and temperature on detecting preplatelet maturation events is explored throughout chapter 4).

To detect preplatelets and barbell platelets directly in whole blood, human anticoagulated blood was labelled with FITC CD61 and 50,000 total BF cellular events (including platelets, RBC and WBC) were acquired by ImageStream^X. As the ImageStream^X lacks traditional scattered light measurements (such as FSC and SSC) used by flow cytometry, cellular events in whole blood were initially defined using BF aspect ratio over area. Aspect ratio divides the major axis of the cell by the minor axis. If a cell is circular it will have a value of 0.8 - 1. If a single cell morphology is elongated or two or multiple cells are imaged together, the aspect ratio range is between 0.6 - 0.2 (depending on the degree of elongatedness). In the BF scatter plot, platelets were identified by interrogating multiple vertically positioned gates (region (R)1-3) spread adjacently across the area axes). From these gates, FITC CD61 fluorescence was interrogated. Platelet structures were only detectable within the “R1” gate (Figure 3.1; mint green). RBC were also present in the R1 gate (Appendix Figure 3.1). Alternatively, region R2 & R3 (magenta and violet respectively) consisted of RBC and WBC only

with some dual RBC events situated in the lower right region of R3 (Appendix Figure 3.1).

FITC CD61 fluorescent images were manually analysed to confirm the largest platelets such as preplatelets and barbells, were present in R1 and therefore not excluded from the analysis. From this, platelets ($<3\mu\text{m}$ in diameter), preplatelets ($>3\mu\text{m}$ in diameter) and barbell platelets were detectable (Figure 3.1). As the largest preplatelets have been reported to be $10\mu\text{m}$ in diameter [380], as expected they were commonly located amongst the RBC population within the R1 gate. No preplatelets were present in gates R2 & R3.

Although ImageStream^X BF imaging was useful to confirm presence of preplatelets and barbells in healthy control whole blood, in order to successfully gate all large platelet structures in haematological conditions associated with macrothrombocytopaenia such as ITP and inherited giant platelet syndromes, the BF gating strategy was abandoned, and 10,000 platelet images were acquired from fluorescence using platelet specific markers (described below).

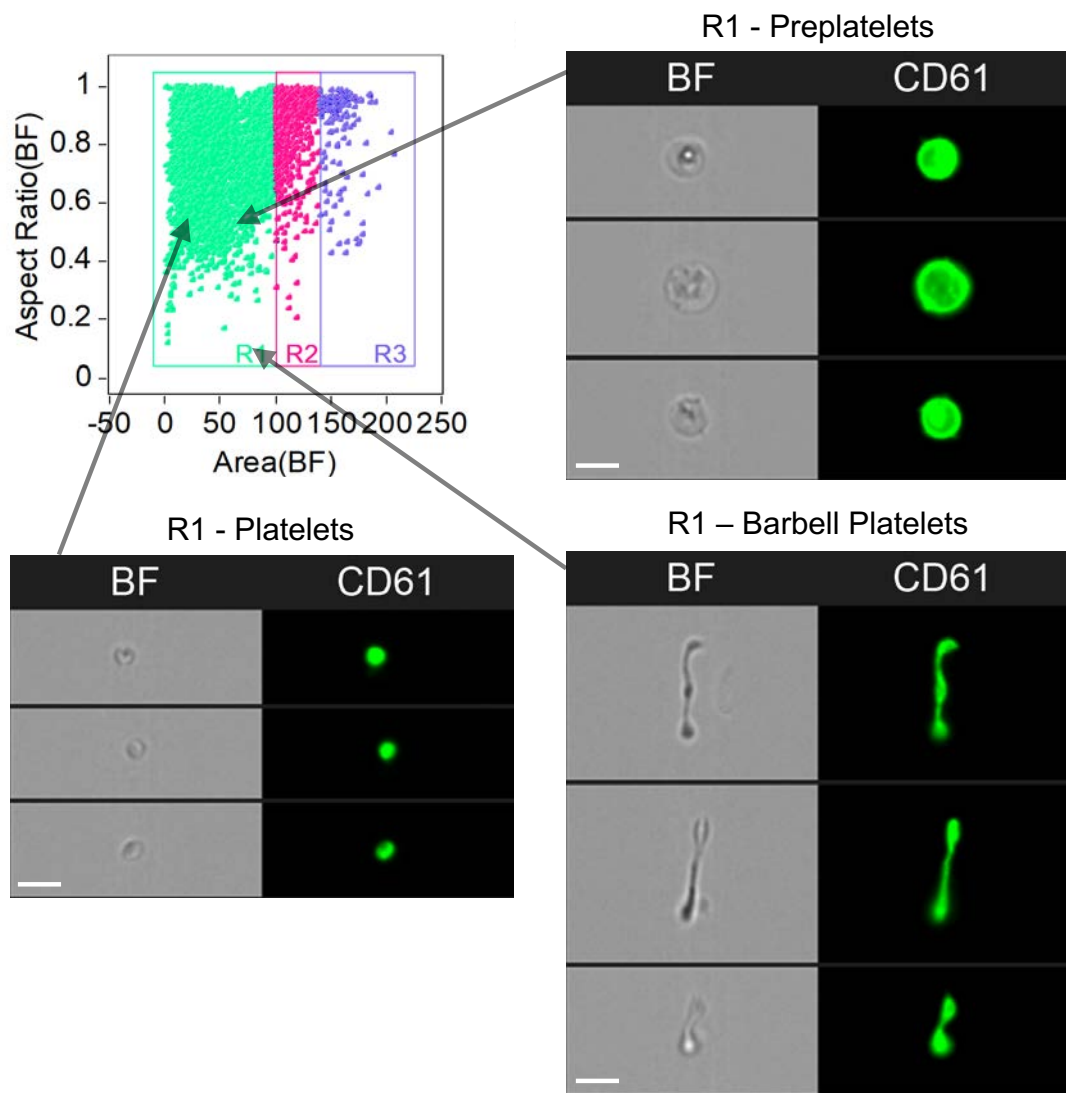


Figure 3.1: ImageStream^X Whole Blood Acquisition

Human whole blood (n=5) was labelled with FITC CD61 and 50,000 brightfield (BF) images were acquired using the ImageStream^X x60 lens under slow flow rate. All whole blood cellular images were displayed using area and aspect ratio of BF images. In this plot, multiple vertically positions gates were generated: “R1” (mint green), “R2 (magenta) & “R3” (violet). Platelet images (depicted by BF and CD61 fluorescence) were only present in the “R1” gate. Platelet images included: platelets (<3 μ m in diameter), preplatelets (> 3 μ m in diameter) and barbell platelets. BF: Brightfield.

Therefore, in order to separate large preplatelets from RBC and WBC, 10,000 FITC CD61/PE CD42b (human) or FITC CD41a only (mouse) platelet images were acquired using a x60 lens under slow flow rate (Figure 3.2a shows an example of the human platelet cluster generated from whole blood). From these 10,000 platelet images, preplatelets and barbell platelets were manually selected and their distribution within the platelet cloud was visualised. As it is hypothesised preplatelets and therefore barbell platelets are large immature platelets, it was unsurprising they were situated within the top region of the platelet cloud (preplatelets determined as yellow dots and barbell platelets by blue dots; Figure 3.2b & 3.2c). However, also situated within this region were circular-shaped platelet clumps (images not shown) and barbell-shaped microaggregates (see Appendix Figure 3.2 for images). However, for barbell-shaped microaggregates, interrogation of FITC CD61 fluorescence alone could not resolve if they were true barbell platelets or two separate platelets bound together. As a result, barbell-shaped microaggregates in whole blood were independently discriminated (Figure 3.12) and further characterised in chapter 4 (Figure 4.1 – 4.5). Thus, morphometric analyses were essential to resolve preplatelets and barbell platelets from the normal platelet cloud.

Platelets, preplatelets and barbell platelets were further defined within each individual whole blood acquisition. The subsequent sections below, describe individually how these populations are fully discriminated with Figure 3.8 and 3.10 combining the approaches to demonstrate the gating strategy in its entirety in human and mice respectively. Once the methodologies were optimised in humans they were confirmed in mice.

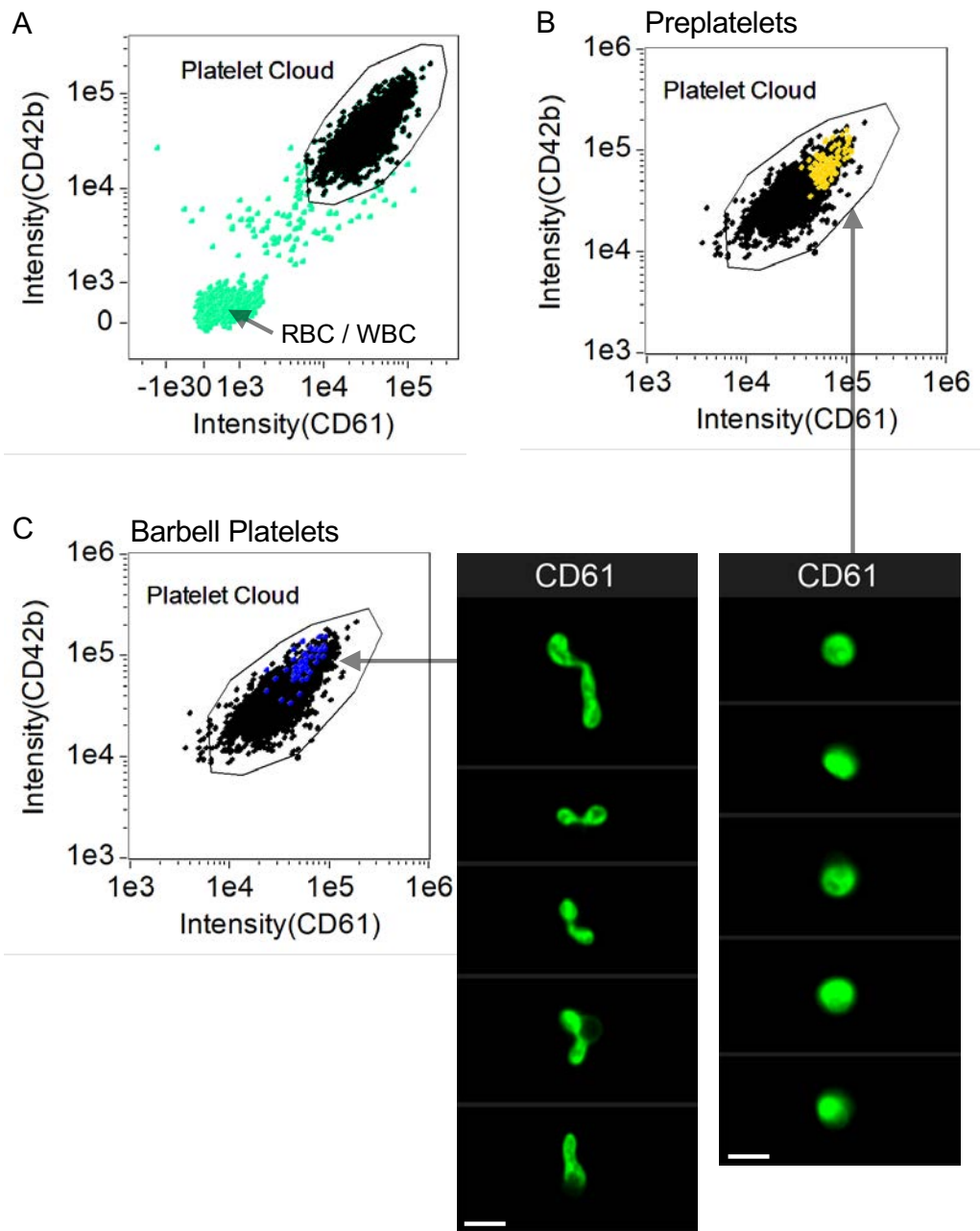


Figure 3.2: location of Preplatelet Maturation Events within the Platelet Cloud

(A) 10,000 FITC CD61/PE CD42b human platelet images were acquired using the ImageStream^x x60 lens under slow flow rate to generate a platelet cloud. (B) preplatelets (yellow dots) and (C) barbell platelets (blue dots) were manually selected by eye to determine their location within the platelet cloud. ImageStream^x images of preplatelets and barbells are depicted by FITC CD61 fluorescence. Scale bar 7 μ m. RBC: red blood cells; WBC: white blood cells.

3.3.2 Determining Preplatelets in Whole Blood Using ImageStream^X IDEAS

Initially, preplatelets in human whole blood were discriminated from the above FITC CD61/PE CD42b positive platelet cloud, using morphometric measurements based on circularity and size. Thon and colleagues (2010) showed preplatelets are identified as large platelet precursor cells 3-10 μ m in diameter [380, 384]. Using laser scanning cytometry to image tubulin labelled human PRP, these researchers demonstrated the minimum barbell platelet perimeter equates to 3 μ m in diameter thus, it is predicted platelets with a diameter below this threshold do not exhibit the ability to convert into barbells and undergo fission [384]. Alternatively, the highest barbell perimeter equated to 10 μ m. Mathematical modelling based on platelet size and microtubule bundling performed by Thon *et al* (2012) suggests preplatelets above this size threshold would contain too many microtubule bundles resulting in the formation of larger than normal platelets [384]. Furthermore, those below the 3 μ m threshold cannot overcome elastic bending forces and cortical tension to elongate and form barbell structures.

Taking the above into consideration, from the FITC CD61/PE CD42b platelet cloud (Figure 3.2a), a CD61 morphology mask was used to depict circularity of a platelet images (Figure 3.3a). Alternatively, a CD61 erode mask plus 4 pixels (erode+4) was designed to exclude background fluorescence and accurately measure platelet diameter (Figure 3.3b). To avoid the inclusion of large circular-shaped platelet clumps, CD62p positive platelet images were excluded from the analysis (Figure 3.3c). From the CD62p negative platelet population, single circular CD61 focussed platelet images were initially discriminated using aspect ratio (0.8-1) and area of a morphology mask (Figure 3.3c) generating the “Circular Platelets” gate. Using the CD61 erode+4 mask, preplatelets were determined by a diameter of 3-10 μ m (Figure 3.3c).

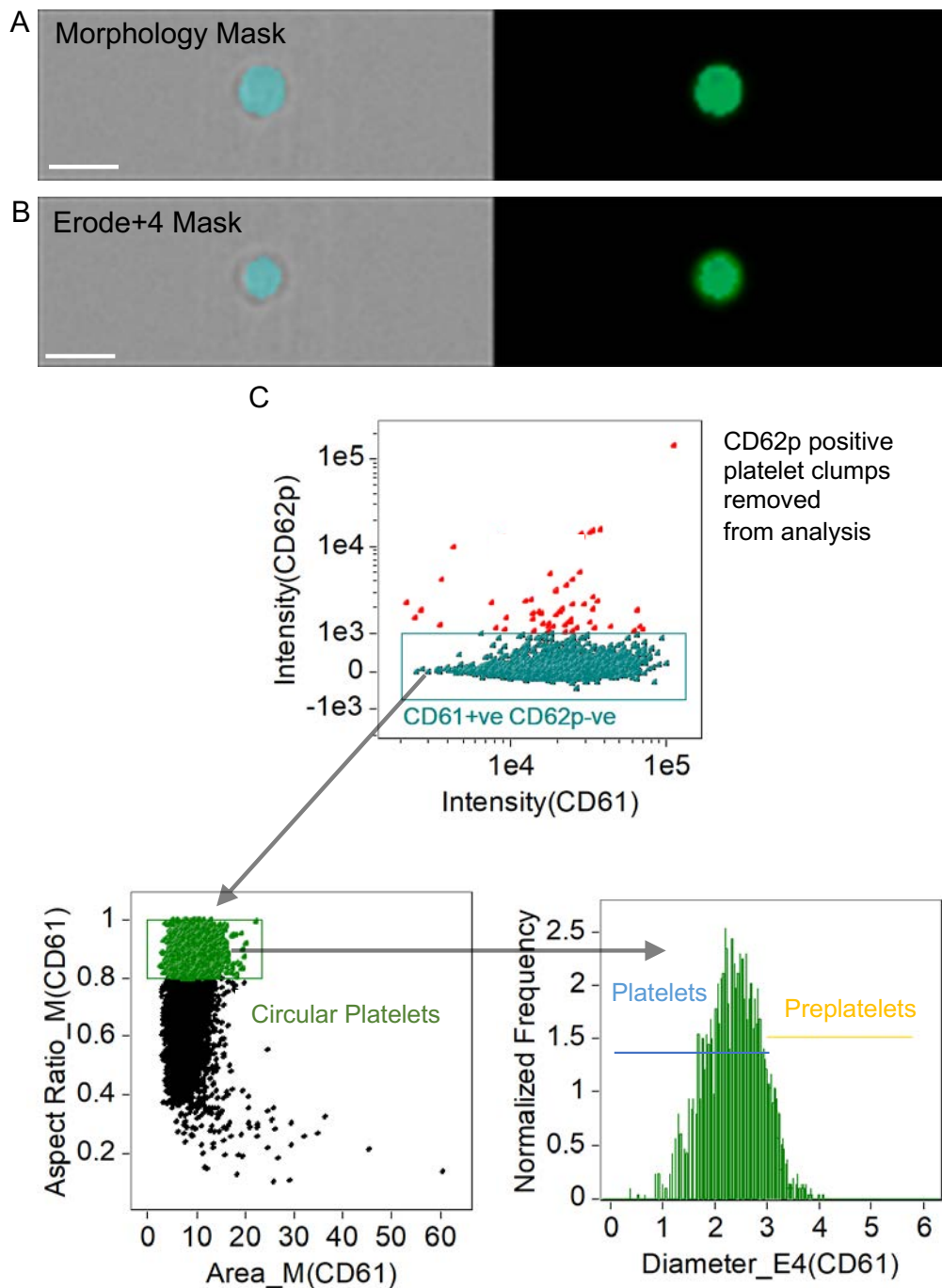


Figure 3.3: Determining Preplatelets by ImageStream^x IDEAS

Example of a (A) morphology mask to exclude the outer contour of a FITC CD61 positive platelet image and an (B) erode mask +4 pixels to exclude 4 pixels from all edges of the default mask (grey: BF; green: CD61; cyan: mask). (C) From 10,000 FITC CD61/CD42b positive platelet cloud, BV421 CD62p positive platelet images were excluded. From the CD62p negative population, circular CD61 platelet images were gated by aspect ratio (0.8 – 1) and area of a morphology (M) mask. Platelets (light blue; <3 μ m in diameter) and preplatelets (yellow; >3 μ m in diameter) were discriminated and quantified from the “Circular Platelets” population using an erode+4 mask (E4) with the feature diameter. Scale bars 7 μ m.

Representative images of platelets and preplatelets generated using the ImageStream^x (x60 lens) are displayed in Figure 3.4a & b respectively. Images are depicted by BF and CD61 fluorescence.

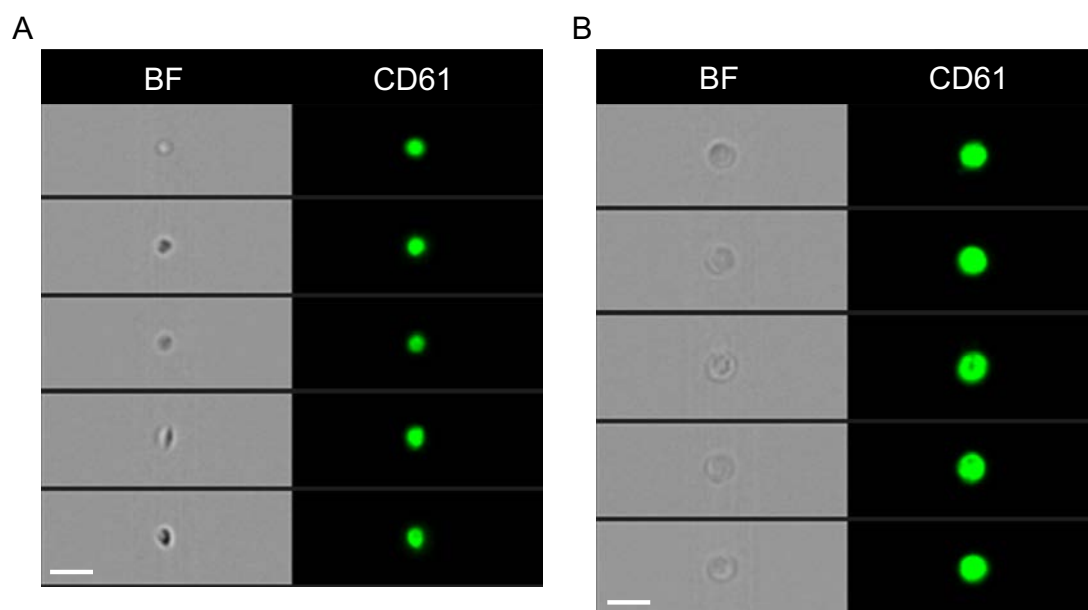


Figure 3.4: ImageStream^x Galleries to Depict Morphology of Platelets and Preplatelets
Examples of gated (A) platelets (<3 μ m in diameter) and (B) preplatelets (>3 μ m in diameter) determined by brightfield (BF) and FITC CD61 fluorescent images using ImageStreamX x60 lens. Scale bars 7 μ m.

3.3.3 Discriminating Barbell Platelets Using ImageStream^x IDEAS

Barbell platelets (see Figure 3.2 and Appendix 4.2 for images) were discriminated from the same FITC CD61/PE CD42b positive platelet cloud displayed in Figure 3.2a and as used above for the preplatelet analysis (Figure 3.3c). Like the preplatelet analysis, CD62p positive events were also excluded to avoid barbell-shaped microaggregate contamination within the final barbell gate (see Figure 4.1 - 4.5 for complete characterisation of barbell-shaped microaggregates). As barbell platelets were

distributed throughout the top half of the platelet cloud (Figure 3.2c; blue dots), morphometric analyses were essential for their complete resolution.

To determine the appropriate morphology features (see Table 2.7 for examples) to discriminate single discoid or circular platelets (singlets) from elongated barbell platelets (“barbells”), truth populations consisting of 100 singlets” and “barbells” were manually selected from the CD61 fluorescence images (Figure 3.5a). Morphology and skeleton thin pixel masks were created to depict the overall shape of an object and locally within a single pixel thick internal cytoskeletal line extending vertically through an image respectively (Figure 3.5b & 3.5c shows examples of these masks respective to singlet and barbell truth populations). Using the truth populations and masks, in combination with feature categories size, location, shape and texture (see Table 2.7) median RD reports were generated (Table 3.1 displays an example of the top 15 features with the highest median RD values based on a CD61 morphology mask).

Median RD reports were generated individually for the morphology and skeleton thin mask. Unfortunately, the probability of the top two features from these tables accurately discriminating respective truth populations is low. Instead, features were logically selected to best depict the barbell shape. For example, from the RD report in Table 3.1 (generated from a morphology mask based on CD61 fluorescence), features such as symmetry2 (the tendency of an object to consist of a two-lobed symmetrical axis), height, compactness (the degree in which pixel intensity is compact), minor axis intensity (shortest diameter of the CD61 morphology mask weighted by fluorescence intensity) and area were chosen. Alternatively, from the RD report generated from a skeleton thin mask (based on FITC CD61 fluorescence), the feature major axis

intensity (longest diameter of the CD61 skeleton thin mask weighted by intensity) was utilised.

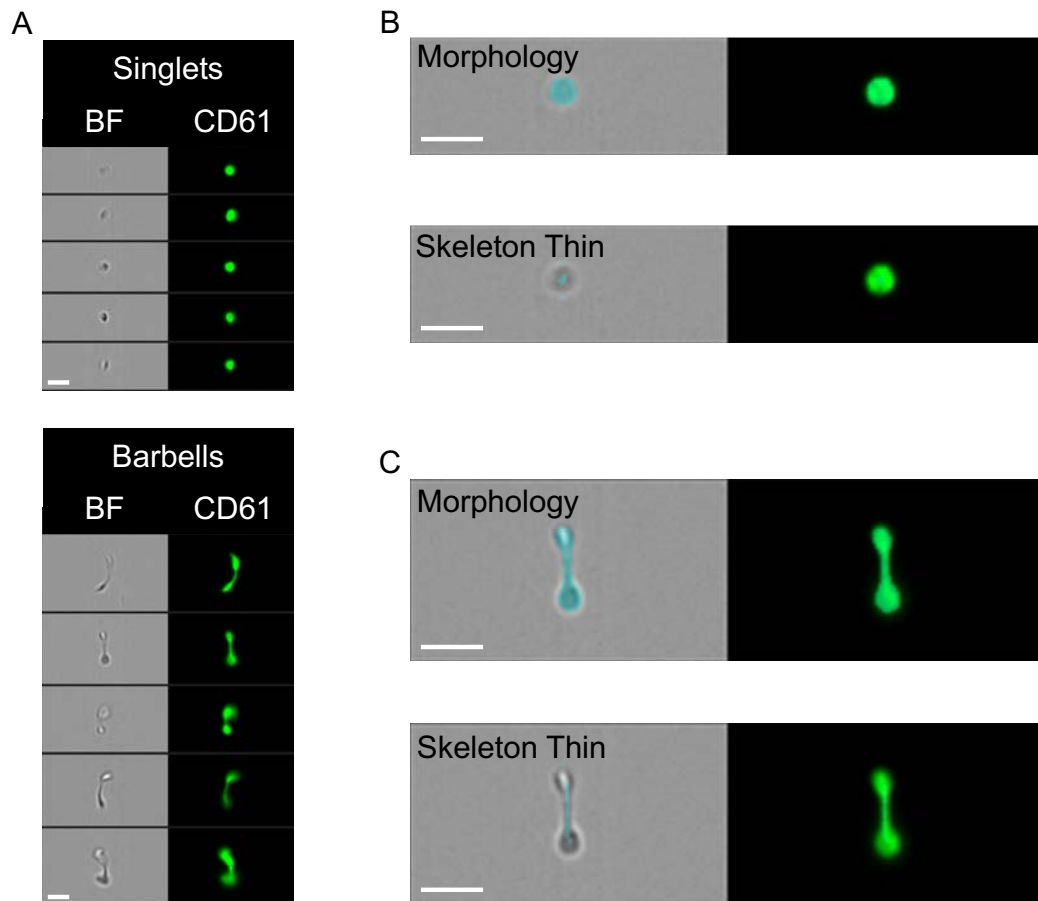


Figure 3.5: Truth Populations with Respective Pixel Masks

From the FITC CD61/PE CD42b positive platelet cloud, (A) truth populations consisting of singlet and barbell platelet images were manually selected based on CD61 fluorescence (images are depicted by brightfield and CD61 fluorescence). Examples of pixel masks morphology and skeleton thin (based on CD61) depicted within (B) singlet and (C) barbell truth populations. Images are depicted by brightfield (grey), FITC CD61 (green) and masks (cyan). Scale bars 7 μ m.

Table 3.1 Median RD Statistics Report Generated from Singlet and Barbell Truth Populations and a FITC CD61 Morphology Mask (Listed in Descending Order)

Feature/Mask/Truth Population	RD
Aspect Ratio_Morphology(CD61) RD - Median, Barbell, Singlets	3.8303
Symmetry 2_Morphology(CD61) RD - Median, Barbell, Singlets	3.323
Aspect Ratio Intensity_Morphology(CD61) RD - Median, Barbell, Singlets	3.1555
Major Axis_Morphology(CD61) RD - Median, Barbell, Singlets	2.7222
Major Axis Intensity_Morphology(CD61) RD - Median, Barbells, Singlets	2.5612
Circularity_Morphology(CD61) RD - Median, Barbells, Singlets	2.2643
Height_Morphology(CD61) RD - Median, Barbells, Singlets	2.2483
Length_Morphology(CD61) RD - Median, Barbells, Singlets	2.2483
Compactness_Morphology(CD61) RD - Median, Barbells, Singlets	1.8364
Perimeter_Morphology(CD61) RD - Median, Barbells, Singlets	1.7986
Elongatedness_Morphology(CD61) RD - Median, Barbells, Singlets	1.7624
Shape Ratio_Morphology(CD61) RD - Median, Barbells, Singlets	1.7175
Area_Morphology(CD61) RD - Median, Barbells, Singlets	1.6862
Spot Area Min_Morphology(CD61) RD - Median, Barbells, Singlets	1.6862
Diameter_Morphology(CD61), RD - Median, Barbells, Singlets	1.5745

RD: Relative Difference

Using an amalgamation of the CD61 morphology features, it was possible to discriminate all elongated platelet structures from the CD61 platelet population, plotting the feature compactness against combined features symmetry2 multiplied by height (Sym2*H; Figure 3.6a). From this scatter graph, 3 regions were gated: compact (high compact and low Sym2*H; black), uncertain (intermediate compact and low-intermediate Sym2*H; red) and elongated platelets (intermediate-low compact and intermediate-high Sym2*H; purple).

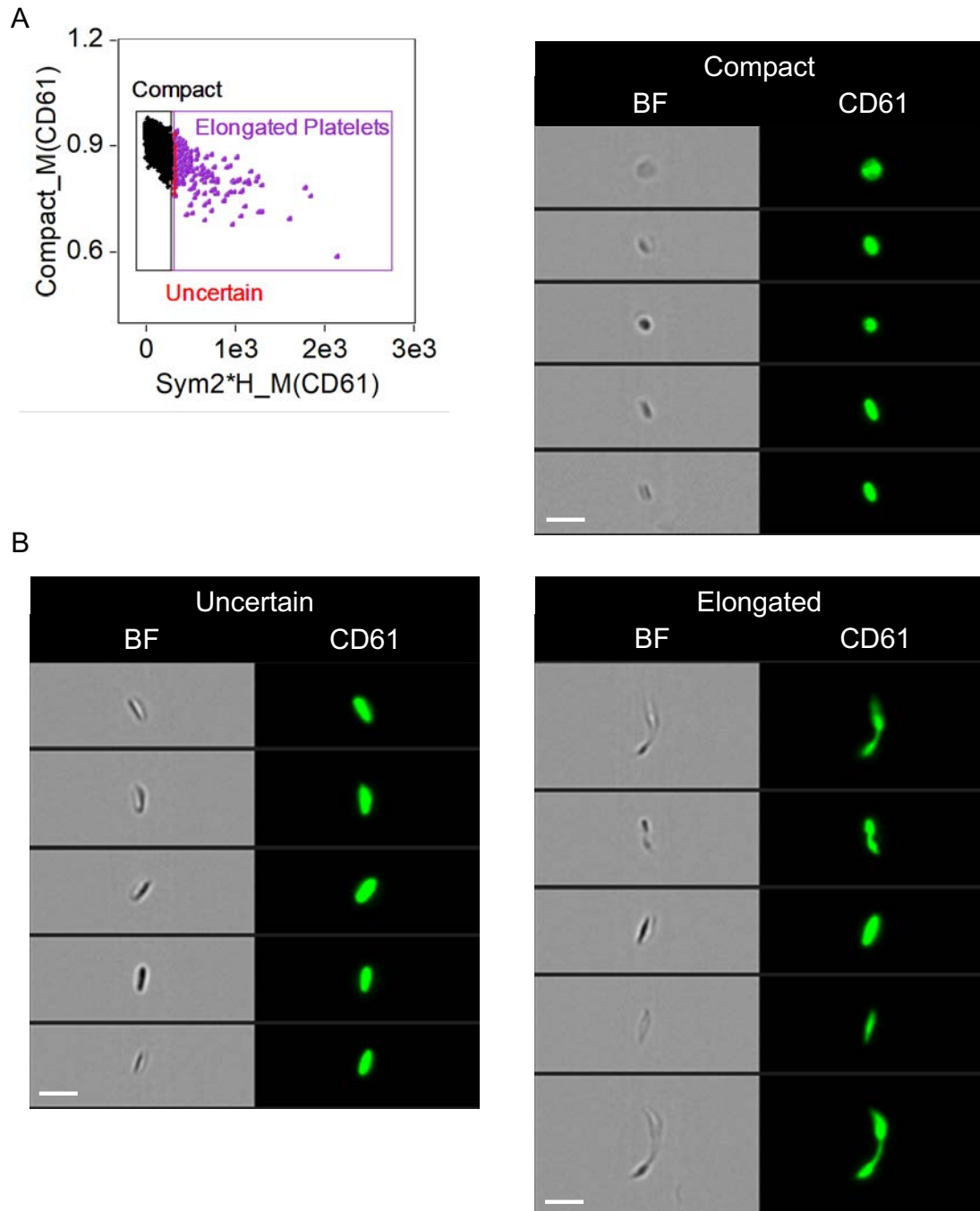


Figure 3.6: Discriminating Elongated Platelet Structures from Normal Platelets

(A) Using the truth populations singlets and barbells, in combination with a CD61 morphology mask, elongated platelet structures were discriminated from compact circular/discoid platelets plotting the feature compactness (compact_M(CD61)) against combined features symmetry2 multiplied by the height of CD61 morphology (Sym2*H_M(CD61)). From this scatter plot, 3 regions were gated: compact (black), elongated platelets (purple) and uncertain (red). (B) ImageStream^X imagery depicting brightfield (BF) and CD61 images of compact, uncertain and elongated platelet populations. Scale bars 7 μ m.

The compact population consisted of circular and discoid platelets (Figure 3.6b). In contrast, the elongated platelet population contained a mixture of discoid, small elongates and barbell platelets. To accurately define elongated platelets from other platelets, a thin vertical gate was positioned adjacent to the elongated platelets gate termed “uncertain” and images from this region were analysed manually by eye to ensure no elongated barbell structures were excluded from the analysis.

For definitive barbell platelet discrimination, both FITC CD61 morphology and skeleton thin features were used. Barbell platelets were subsequently separated from the “elongated platelet” population in Figure 3.6a using minor axis intensity of CD61 morphology against the area of CD61 morphology multiplied by the major axis intensity of CD61 skeleton thin (Figure 3.7a). As barbell platelets consist of a two distal platelet structures interconnected by a thin cytoplasmic bridge [380, 382] (Figure 3.7b), these structures were separated from non-barbell structures (discoid platelets; Figure 3.7b) by a high minor axis and symmetry² score (Figure 3.7a). Also, elongated preplatelets which had not yet formed a barbell-shape contained a medium to high minor axis of CD61 morphology with a low major axis skeleton thin value (Figure 3.7c). CD62p negative platelet coincidence events were also present however, these were rare events. The location of all 3 structures within the barbell gate can be viewed in Figure 3.6d (barbell platelets (blue), elongated preplatelets (green), coincidence events (red)). In contrast, the non-barbell population consisted of small elongated discoid platelets.

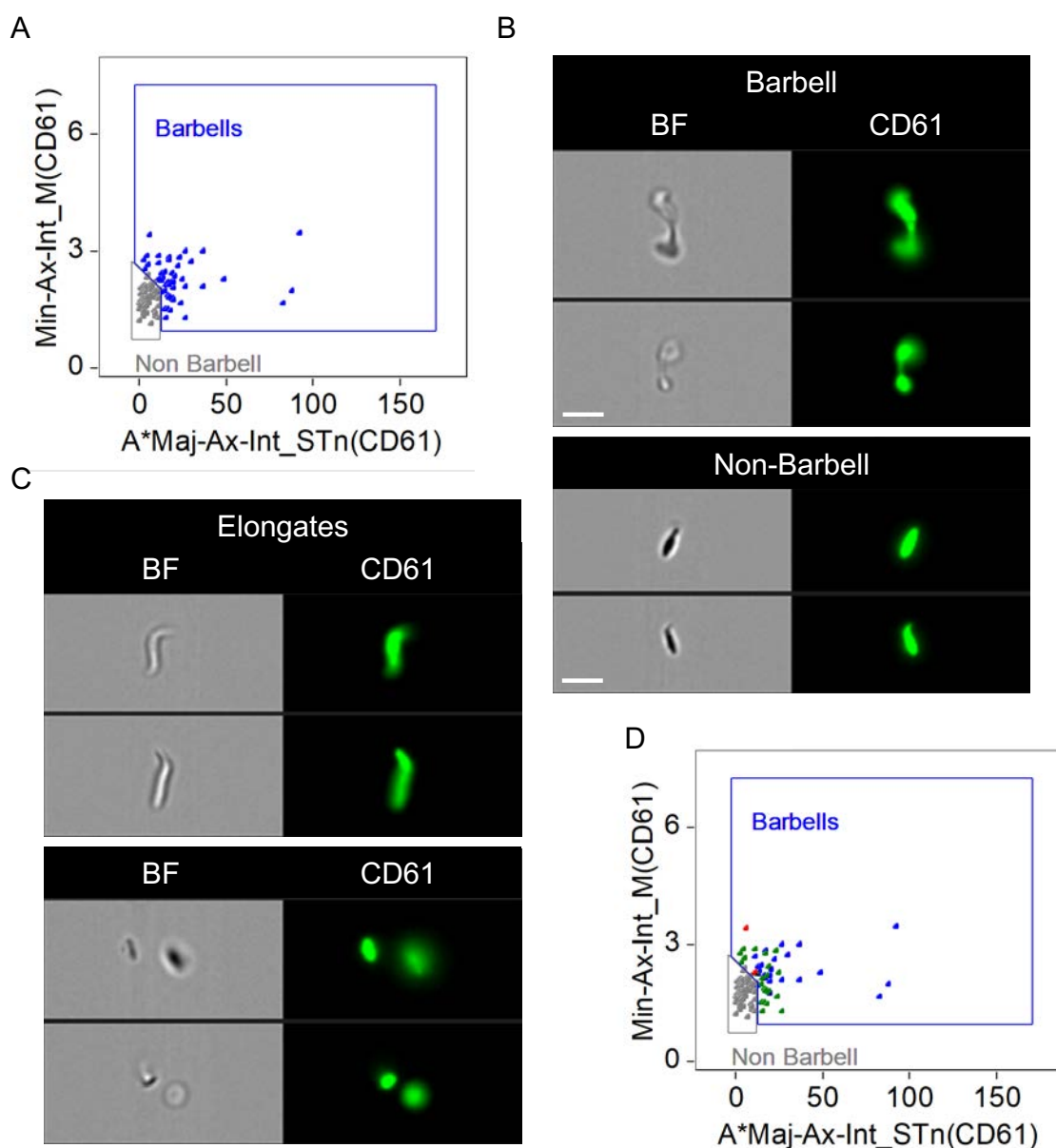


Figure 3.7: Discriminating Barbell Platelets

(A) Using ImageStream^X IDEAS, barbell platelets were identified within the elongated platelet population (see Figure 3.6a) by plotting minor axis intensity of CD61 morphology (Min-Ax-Int_M(CD61)) against area of CD61 morphology multiplies by the major axis intensity of CD61 skeleton thin (A*Maj-Ax-Int_STn(CD61)). Barbells are situated in the blue gate and non-barbells in the grey. (B) ImageStream^X images of barbell platelets and non-barbell discoid structures and (C) elongated preplatelets and platelet/platelet coincidence events (depicted by brightfield and CD61 fluorescence; scale bar 7 μ m). (D) localisation of barbell platelets (blue), elongated preplatelets (green) and coincidence events (red) situated within the “barbells” gate.

The complete platelet, preplatelet and barbell platelet ImageStream^X IDEAS gating strategy is displayed below in Figure 3.8.

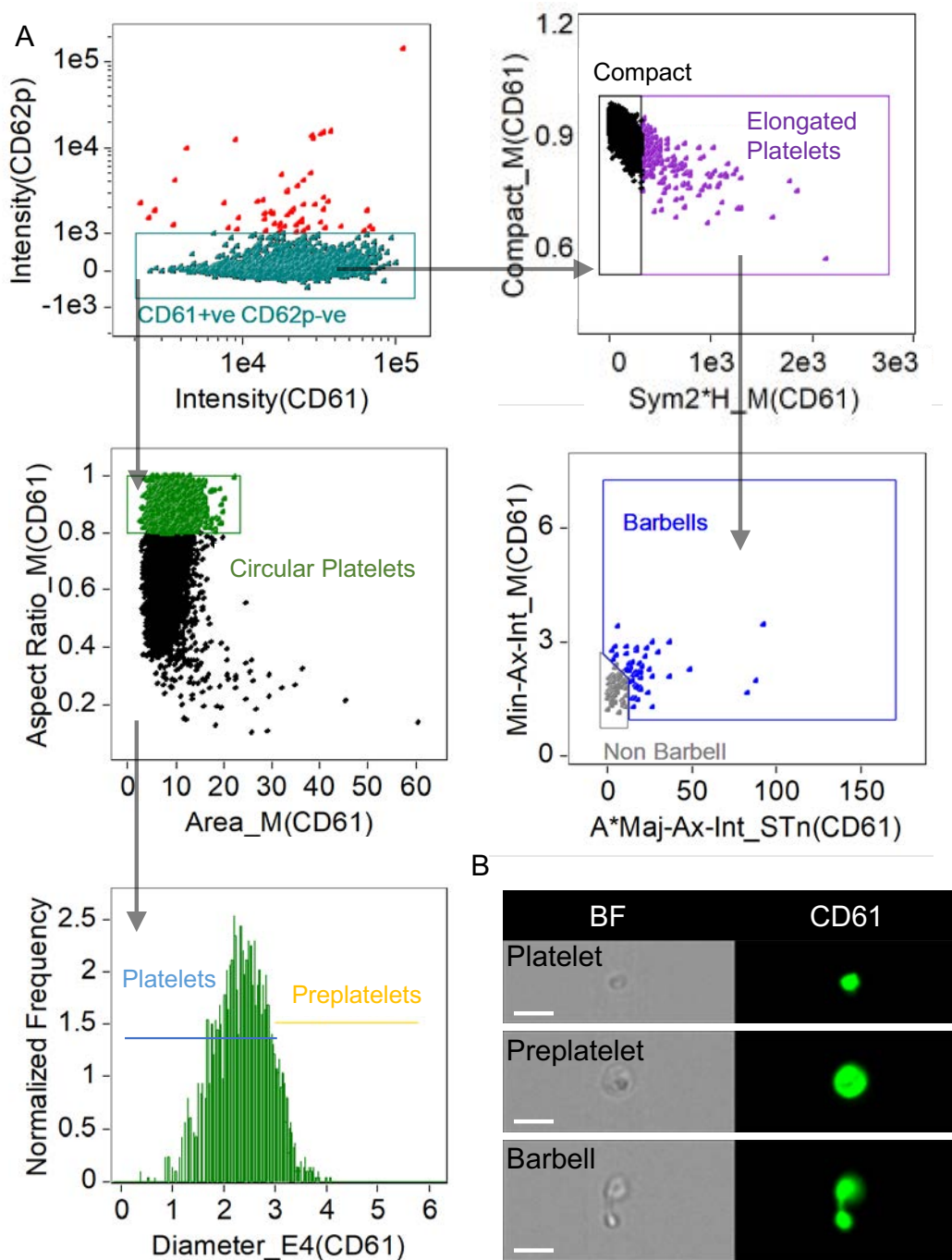


Figure 3.8: Quantifying Preplatelets and Barbell Platelets using ImageStream^x

Platelets, preplatelets and barbell platelets were quantified from the FITC CD61/PE CD42b positive platelet cloud (Figure 3.2a). (A) CD62p positive platelets (including barbell-shaped microaggregates) were excluded from the analysis. For preplatelets (left side; see Figure 3.3 for full description). For barbell platelets (right side): elongated platelet structures were initially separated from single circular or discoid platelet images using compactness and symmetry2 multiplied by height (Sym2*H) of CD61 morphology. Elongated platelets were further interrogated by minor axis intensity (Min-Ax-Int) CD61 morphology against area multiplied by major axis intensity (A*Maj-Ax-Int) CD61 skeleton thin (STn) generating the “barbells” region. (B) ImageStream^x images (x60 lens) of platelets, preplatelets and barbell platelets using brightfield (BF) and CD61 fluorescence. Scale bar 7 μ m.

3.3.4 Validating Preplatelet and Barbell Platelet ImageStream^x IDEAS Discrimination

As preplatelets are characterised by a normal yet large marginal band morphology ($>3\mu\text{m}$ in diameter) and barbell platelets an elaborate figure-of-eight morphology [380, 382, 384], we used SiR tubulin labelling of live cells to further validate our ImageStream^x methodologies. Human citrated blood was labelled with BV421 CD62p, FITC CD61 and SiR tubulin ($4\mu\text{M}$, Spirochrome) for 30 minutes at 37°C and platelets, preplatelets and barbell platelets were discriminated as above (Figure 3.8). SiR tubulin labelling successfully labelled platelet marginal bands (Figure 3.9a) and confirmed preplatelets contain a normal marginal band $>3\mu\text{m}$ in diameter whereas, barbell platelets (although limited by resolution) consist of a continuous figure-of-eight tubulin positive cytoskeletal structure (Figure 3.9b – 3.9c). Further investigation regarding marginal band morphology of preplatelet maturation events can be found in Chapter 4 (Figure 4.11 & 4.12).

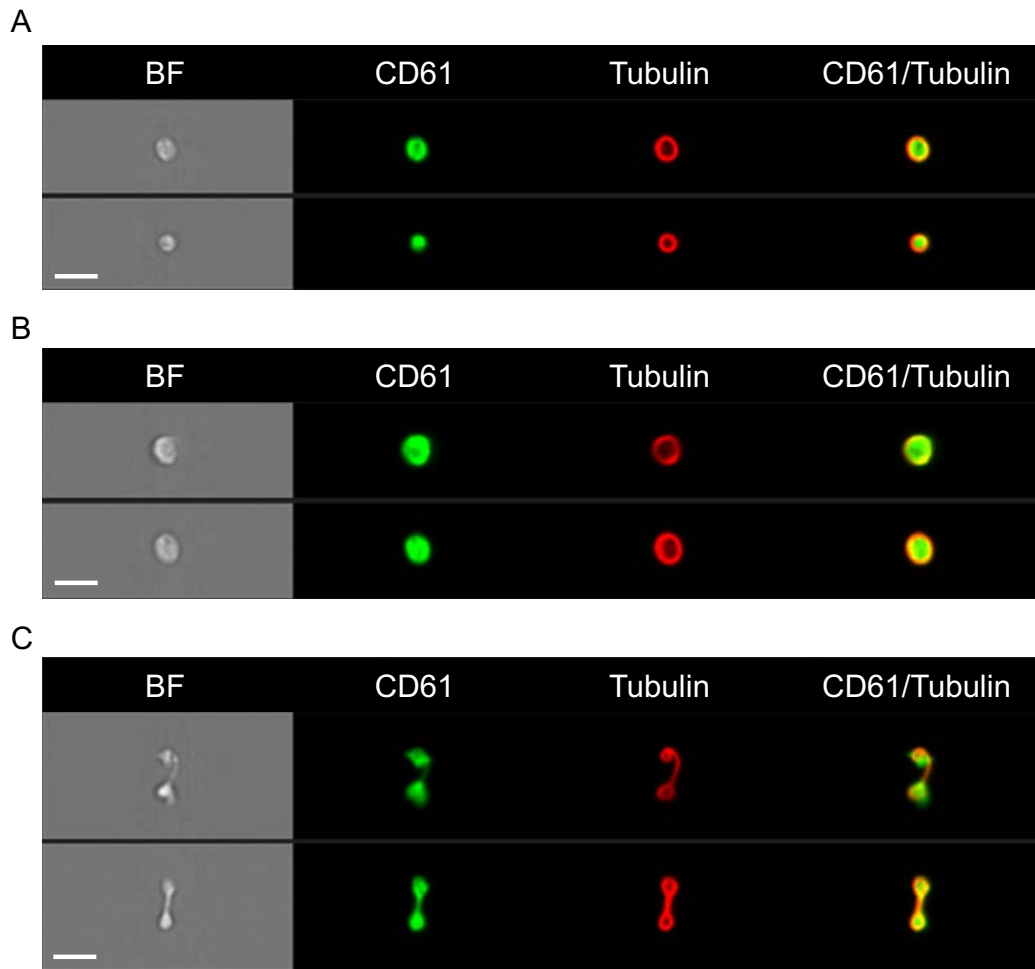


Figure 3.9: SiR Tubulin Labelling of Preplatelets and Barbell Platelets Discriminated by ImageStream^x

Healthy control citrated blood was immediately labelled with BV421 CD62p, FITC CD61 and AF 674 SiR tubulin (4 μ M) for 30 minutes at 37°C and 10,000 CD61 platelet images were acquired using the ImageStream^x. SiR tubulin labelling was used to depict the marginal band of (A) platelets, (B) preplatelets and (C) barbell platelets discriminated by ImageStream^x (x60 lens). Images is representative of a single experiment. n=5. Scale bar 7 μ m. BF: brightfield; Tubulin: SiR tubulin.

3.3.5 Discriminating Preplatelets and Barbell Platelets in Mice

Mouse platelets were determined from 10,000 focussed FITC CD61 positive/BV421 CD62p negative events. See gating strategy below in Figure 3.10. See Appendix 6.1 & 6.2 for additional images of preplatelets and barbells in murine whole blood.

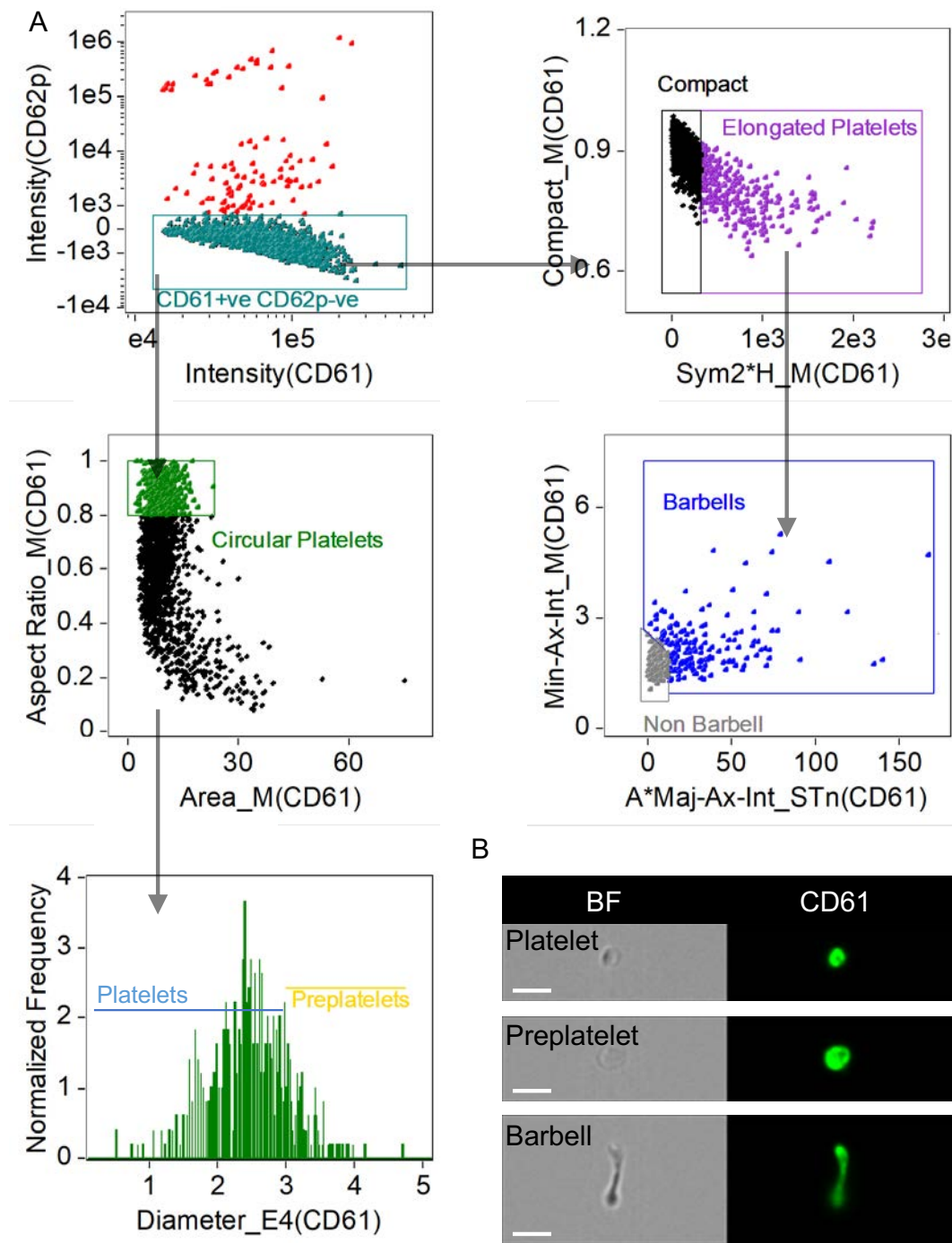


Figure 3.10: Quantifying Murine Preplatelets and Barbell Platelets using ImageStream^x IDEAS

(A) Murine preplatelets and barbell platelets were quantified from 10,000 CD61 positive/CD62p negative platelet images. Preplatelets and barbell platelets were discriminated as described for human blood in Figure 3.8. (B) ImageStream^x images (x60 lens) of murine platelets, preplatelets and barbell platelets using brightfield (BF) and CD61 fluorescence. Scale bar 7μm. M: Morphology; E4: Erode+4; STn: Skeleton Thin; Sym2*H: Symmetry2 * Height; A*Maj-Ax-Int: Area*Major Axis Intensity; Min-Ax-Int: Minor Axis Intensity.

3.3.6 Discrimination of Barbell-Shaped Microaggregates

Preplatelet maturation events in healthy donor citrated whole blood were analysed at room temperature and 37°C (see chapter 4). Although preplatelets and barbell platelets were detectable at 37°C, at room temperature, no barbell platelets containing a thin cytoplasmic bridge (a key characteristic of barbell platelets) were detectable. Instead, only a discrete population of barbell-shaped microaggregates (consisting of two distal platelet structures) were present (full characterisation was conducted in chapter 4; Figure 4.1 – 4.7). To determine whether barbell-shaped microaggregates in room temperature citrate blood were indeed related to true barbell platelets, it was essential to design an ImageStream^X methodology for their accurate discrimination (described here).

Barbell-shaped microaggregates (Figure 3.11a, Figure 4.1 & Appendix Figure 3.2) were quantified using a similar method to barbell platelets. Using the same antibody labelling and ImageStream^X acquisition described above, 10,000 FITC CD61/PE CD42b positive platelets images were acquired from room temperature citrated blood. However, no BV421 CD62p labelling was performed at this stage. Singlet (Figure 3.5a) and microaggregate (Appendix Figure 3.2) truth populations combined with a CD61 morphology mask were used to generate a median RD report to determine the best features for discriminating microaggregates (table not shown; see Table 3.1 for an example of an RD stats report). As microaggregates were also barbell-shaped, they were initially identified within the same “elongated platelets” gate described above. From this gate, microaggregates were distinguished using symmetry2 and intensity of CD61 morphology mask (Figure 3.11b). Microaggregates contained a higher CD61 intensity than singlet platelets with a low to high symmetry2 score. The variation of the

symmetry2 score was dependent on the orientation in which microaggregates passed through the flow cell. Those with a high symmetry2 score consisted of two complete circular platelet structures whereas, low symmetry images consisted of one platelet structure which partially eclipsed the other.

As these microaggregates were barbell-shaped, it was important to determine whether they were situated within the barbell gate developed from 37°C whole blood in the absence of a BV421 CD62p dumping gate. Figure 3.12 demonstrates, when incubating citrate blood at 37°C for 15 minutes, microaggregates were situated in the barbell gate (Figure 3,12; red dots) in the absence of CD62p gating. Importantly, these microaggregates can be removed from the barbell gate through gating out CD62p positive platelet images. Excluding barbell-shaped microaggregates using CD62p fluorescence was a critical discovery to optimise the barbell platelet ImageStream^x analysis in human and mouse 37°C blood (Figure 3.8 & 3.10).

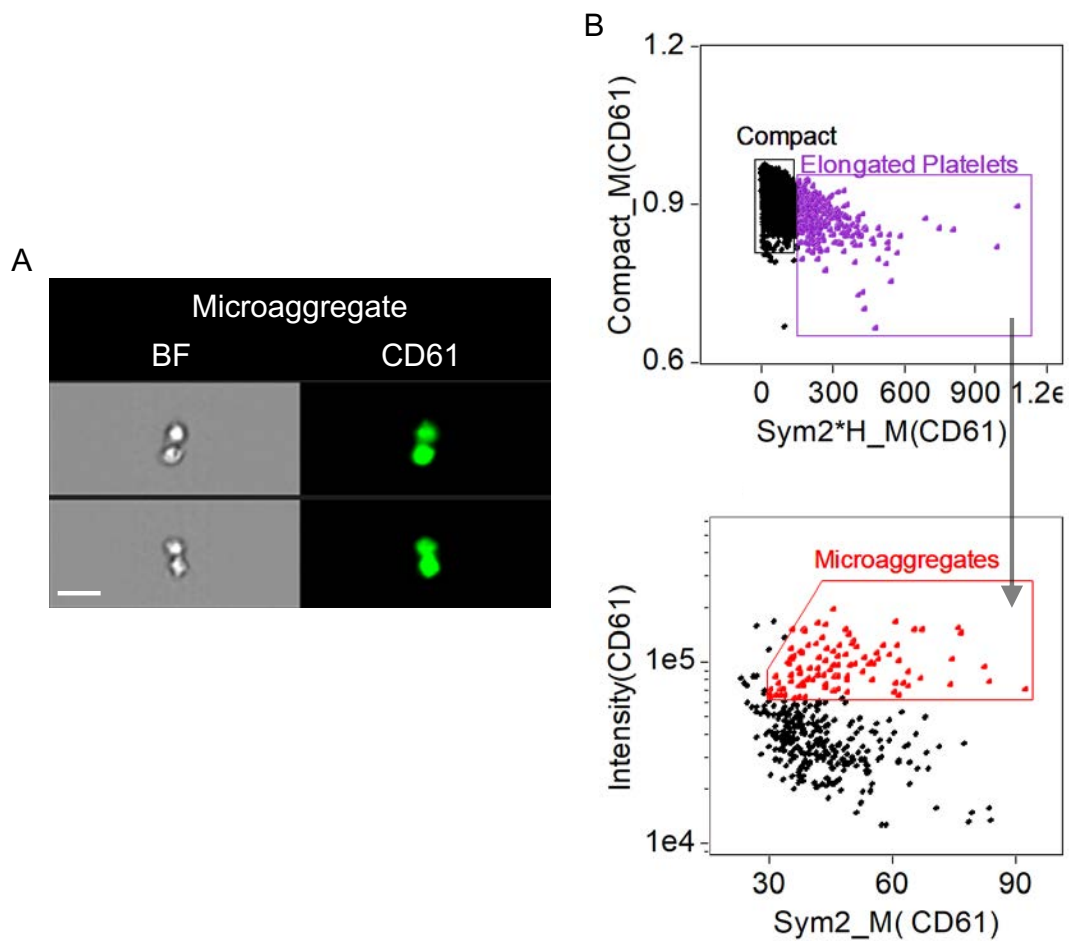


Figure 3.11 Determining Platelet Microaggregates Using IDEAS

(A) ImageStream^X analysis of barbell-shaped microaggregates in room temperature citrate blood depicted by brightfield (BF) and CD61 (scale bar 7 μ m). (B) Using ImageStream^X IDEAS, elongated platelets were discriminated from focussed CD61 positive platelet images. Microaggregates consisting of two distal platelet structures were distinguished from other elongated platelets by symmetry2 CD61 morphology and CD61 fluorescence intensity. M: morphology; Compact: Compactness; Sym2: Symmetry2; H: Height.

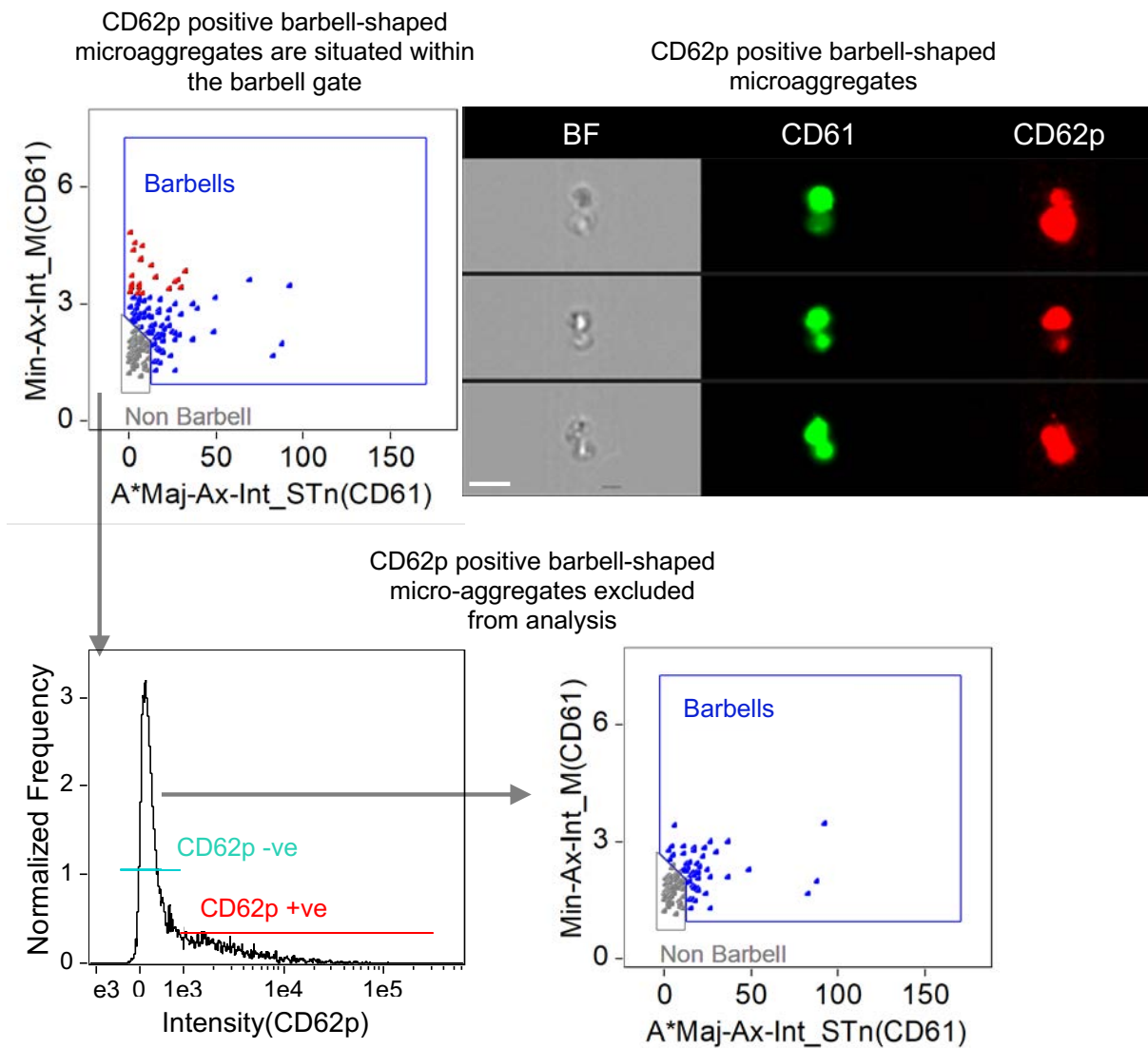


Figure 3.12: Barbell-Shaped Microaggregates are Excluded from the Barbell Gate Through CD62p Fluorescence

In the absence of BV421 CD62p gating, barbell-shaped microaggregates are present within the barbell gate (red dots) in citrate blood at 37°C. As these microaggregates express CD62p (see images), they can be removed from the barbell population by gating out CD62p positive events. Scale bar 7µm. BF: Brightfield; M: Morphology; A*Maj-Ax-Int: Area*Major Axis Intensity; Min-Ax-Int: Minor Axis Intensity.

As the purpose of this chapter is to describe the methodologies used to define the above populations (platelets, preplatelet, barbell platelets and barbell-shaped microaggregates), these methods will be used throughout the thesis to perform a

detailed investigation to accurately characterise and quantitate preplatelet maturation events in healthy and abnormal human and mouse blood samples.

3.4 Discussion and Limitations

With the use of ImageStream^X, we have designed and validated IDEAS methodologies to accurately discriminate preplatelet and barbell platelet events in whole blood using morphometric parameters based on cellular size and shape. Unlike slide-based high-throughput imaging techniques, ImageStream^X consists of a complex hydrodynamic fluidics network thus, making it possible to accurately image cellular events directly from whole blood (Figure 3.1 & 3.2a). Initial observations of the CD61/CD42b platelet cloud in whole blood showed preplatelets and barbell platelets were distributed throughout the top half of the platelet cloud (Figure 3.2b & c). As a result, using CFC to resolve these platelet maturation events is highly challenging. Thus, it was essential to utilise morphometric analyses afforded by ImageStream^X.

Preplatelets are hypothesised to be large platelet precursor cells (3-10µm in diameter) [380, 384]. As a result, preplatelets were quantified based on diameter of CD61 fluorescence (>3µm; Figure 3.3). In contrast, barbell platelet discrimination was more complex than preplatelets and required manually selecting truth populations and masks to generate and logically select appropriate features for the analysis (the same process used for microaggregate discrimination; Figure 3.5 & Table 3.1). However, as barbell platelets consist of a unique and elaborate figure-of-eight morphology [380, 382], it was possible to utilise pixel masks (CD61 morphology and skeleton thin) combined with morphological feature measurements to discriminate barbell platelets

from other platelets based on their elongated extracellular and cytoskeletal features (Figure 3.6 - 3.9).

For both preplatelet and barbell platelet discrimination, it was also essential to exclude CD62p positive events from the analysis as these are found in microaggregates consisting of 2 platelets. Although ImageStream^X is a powerful tool for analysing morphometric cellular features it cannot discriminate between morphologically similar objects. For example, circular-shaped microaggregates appear the same as single circular cellular events. A key limitation regarding the barbell analysis was the contamination of barbell-shaped aggregates (See Figure 3.11, 3.12, 4.1 & Appendix 3.2 for images). Importantly these were gated out from barbell the analysis using CD62p fluorescence (Figure 3.8 & 3.10). Complete characterisation of barbell-shaped microaggregates can be found in chapter 4 (Figure 4.1 - 4.7).

When developing IDEAS analyses it is essential to analyse focussed images and select appropriate pixel masks. For example, although ImageStream^X is an incredibly powerful tool combining flow cytometry and microscopy, it still lacks the resolution afforded by advanced microscopy. As a result, fluorescent images often contain background signal and/or can be partially out of focus. Careful consideration needs to be taken when selecting truth populations to generate features, as out of focus images would yield different features compared to those in focus. Also, background fluorescence signal is dependent on the concentration and type of fluorochrome. Although isotype controls were used to determine positive labelling, this does not remedy the fact some images still consist of a hazy outer contour, due to limitations regarding image resolution. So good standardised fluorescence labelling and image optimisation is essential to generate reproducible data. Thus, measurements of

platelet diameter (in our case preplatelets) are highly dependent on fluorescence background and the subsequent mask we used (erode+4). Furthermore, as barbell platelets contain a thin cytoplasmic bridge, background and therefore mask type could potentially exclude fine detail from the analysis. Thus, careful considerations should be made when designing masks to perform accurate and efficient analyses. To overcome such limitations, we performed visual validation of masks by inspecting the region in which each mask covered. Using brightfield imagery we created a new view where separate columns represented different masks. From this we concluded morphology, skeleton thin and erode+4 pixels were the most appropriate for designing features to conduct our analyses. This validation method has been shown to be highly useful for selecting masks [390].

The number and quality of any selected truth populations is also important. For example, in our study, truth populations were manually selected from samples which contained an abundance of barbell platelets. The IDEAS manual suggests >20 cellular images are required for generating adequate features to discriminate truth populations. However, we found when comparing median RD reports consisting of truth populations containing either 20 or 100 cellular images can yield different feature tables (data not shown).

Although we have designed and validated an ImageStream^X method for preplatelet and barbell platelet quantitation in whole blood, during this process, multiple feature parameters were investigated manually by using the “Feature Finder” wizard (an inbuilt systematic approach which automatically finds the best feature for the analysis based on the masks and truth population selected by the user). This approach yielded different results varying in efficacy (particularly for barbell platelet analyses). From this,

almost trial and error process, we decided upon using the manually developed methodologies described above. However, a key consideration is methods can also differ depending on the user and often, different approaches can result in equally as good results. This highlights the importance of validating the masks that were used. Once the methodologies were defined, we confirmed marginal band morphology of preplatelets and barbell platelets using SiR tubulin (Figure 3.9). SiR tubulin is a non-toxic fluorogenic probe which crosses the membrane. This probe has been shown to be superior at labelling the microtubules of living cells [420]. Thus, originally, we explored whether this probe could be used as an alternative or in addition to FITC CD61 fluorescence to better discriminate and quantify preplatelets and barbell platelets in whole blood. One other study discriminated preplatelets and barbell platelets in humans using laser scanning cytometry imaging of tubulin labelled platelets [384]. However, this method required PRP separation and cell permeabilization to perform imaging of microtubules. SiR tubulin has the potential to avoid any such preparation steps, removing unnecessary variables and potential artefacts from the analysis. Unfortunately, due to the high concentration and heterogeneity of cells in whole blood (such as RBC), SiR tubulin labelling required a concentration 4x greater than the manufacturer's recommendations (4 μ M instead of 1 μ M). At 4 μ M, SiR tubulin labelling was able to label some platelets successfully but often the signal was saturated in other platelets. Unfortunately, use of concentrations below this threshold yielded very weak or sometimes absent signals. Although we titrated this probe with or without an efflux pump inhibitor verapamil (10 μ M; a general efflux pump inhibitor known to improve SiR tubulin labelling efficacy) the optimal concentration and incubation time could not be fully determined. As a result, we were only able to

generate representative platelet, preplatelet and barbell platelet images and not label the complete platelet population successfully. Despite this we could still use this approach to validate the morphology of the platelet populations.

3.5 Conclusions

Using ImageStream^x and IDEAS, we have designed and validated high throughput image-based methodologies to quantitate platelets, preplatelets and barbell platelets in whole blood. In doing so, we have carefully excluded similarly shaped microaggregates caused by artefactual platelet activation. In proceeding chapters, we will use these methods to now characterise and quantify platelet populations and determine the physiological relevance of preplatelet maturation in normal and thrombocytopenic blood.

**Chapter 4 - Detecting and
Characterising Preplatelet Maturation
Events in Healthy Human Whole
Blood**

4.1 Introduction

The youngest circulating platelets are historically known as RP [5]. RP were first described in a canine model of acute blood loss and were characterised by their greater size and RNA content [349]. Using nucleic acid dyes (e.g. thiazole orange), RP can be measured by flow cytometry [353]. Using this method, it is possible to distinguish between thrombocytopenic conditions with enhanced peripheral destruction or lack of production with potential to monitor BM recovery following transplantation [361, 365]. However, due to poor standardisation and problems with dye specificity, the method largely remains a research tool and has yet to translate into routine clinical practice. Furthermore, no specific antigenic markers of RP exist that facilitate their complete resolution from mature platelets.

Although our understanding of platelet production has improved significantly over recent decades, details of the final stages of platelet biogenesis remain controversial and elusive. Although the consensus view was that BM MK release platelets in a terminally differentiated state, [151, 152] *in vivo* imaging of BM MK demonstrated that they in fact release large cytoplasmic structures or proplatelets into the sinusoid vessels suggesting that platelet maturation events continue within the bloodstream [163, 174, 380, 381] and the pulmonary circulation [187, 200]. Furthermore, washed platelet concentrates have been shown to transform into proplatelet-like barbell structures (barbells: consisting of an elaborate tubulin figure-of-eight cytoskeleton) *in vitro*, which is accompanied by an increase in biomass and enhanced protein synthesis. Barbell platelets could then also undergo fission into two smaller platelets, a process which increased the platelet count in culture [382]. These newly formed platelet progeny are ultrastructurally and functionally like normal blood platelets. This

led to the discovery of a new terminal stage of platelet maturation which takes place in the bloodstream through a platelet intermediate the “preplatelet” [380].

Circulating preplatelets are large platelet precursors (3-10 μm in diameter) which duplicate by converting into barbells before undergoing fission to form two smaller platelets (2-3 μm in diameter; see diagram in Figure 1.4) [380, 382]. Preplatelets and barbell platelets have both been identified in human PRP using a microscopy-based laser scanning cytometry approach (3.6% and 0.05% respectively) and importantly when transfused into mice have been shown to increase the platelet count 300% greater than standard platelet transfusions [380, 384]. Thus, although it seems likely that preplatelets represent newly formed RP, this has yet to be definitively proven.

There are also a few commercially based RP methods, including the IPF, that are incorporated into routine full blood counters [362, 363]. However, although these measurements are standardised and have good inter-laboratory reproducibility, they are still of limited value as they fail to discriminate RP from large platelets due to non-specifically labelling of nucleotides. As a result, they can exaggerate the rate of platelet production in conditions associated with macrothrombocytopaenia and BM suppression/failure and therefore could potentially misdiagnose thrombocytopenia [421, 422].

Barbell platelets in whole blood have been rarely observed possibly because of limitations with existing technologies. For example, IPF and blood film analysis tend to use EDTA anticoagulated blood. This would not only cause platelet swelling but cause irreversible conversion of platelet barbells into preplatelets [384]. Furthermore, quantification by microscopy is a laborious technique and highly subjective. Thus, it is essential to develop image-based methods such as IFC to characterise, quantify and

understand the physiological relevance of circulating preplatelet-derived barbells in whole blood (see chapter 3 for a full description of IFC methodologies used in this chapter).

The aim of this chapter is to:

- 1) Define the optimal conditions for quantifying preplatelets and barbells by IFC.
- 2) Determine the effect of pre-analytical variables e.g., anticoagulant, time delay and temperature on platelet morphology in whole blood.
- 3) Characterise marginal band morphology during preplatelet and barbell platelet conversion using IFC and advanced microscopy.
- 4) Determine whether preplatelets are equivalent to RP and thus represent the rate of thrombopoiesis.

4.2 Methods

All experiments were conducted with platelets obtained from control human whole blood. Healthy controls were recruited from UOB and aged between 18 and 60 with no known underlying healthy issues. All blood samples were analysed on the XN1000 haematology analyser (Sysmex), generating a full blood count with platelet count, MPV and IPF% to confirm that samples were normal (Appendix Table 4.1). Primarily, whole blood from the same donor was anticoagulated in EDTA, hirudin or 3.2% trisodium citrate (1:10th volume) and 25µL was labelled with 25µL of PBS containing anti-human FITC CD61 (1/20) and PE CD42b (1/50) for 15 minutes at either room temperature

(21°C) or 37°C and fixed in 50µL of 4% formalin (0.22µm) in PBS at respective temperatures prior to ImageStream^X (IFC) acquisition. Using the ImageStream^X acquisition protocol described in chapter 3 (Figure 3.2), 10,000 FITC CD61/PE CD42b platelet images were acquired (n=5). From this platelet cloud, platelet morphology was depicted using aspect ratio (measures the circularity of an image: 1 = circular and 0 = elongated) and area of a FITC CD61 morphology mask at room temperature or 37°C. Blood film analysis (performed using ReastainTM Diff Quik Modified Giemsa Staining kit (see Figure 2.1), aired dried and mounted in DPX mountant; n=3-5) were also used to determine platelet morphology and presence of barbell platelets in whole blood. From here on citrate anticoagulation was used for investigating preplatelet maturation events in whole blood. To ensure accurate barbell structure quantification, the possibility of microaggregate formation was also determined to ensure that these were eliminated from analysis. For barbell-shaped microaggregate characterisation, they were accurately discriminated using an ImageStream^X IDEAS gating strategy (see Figure 3.11) under room temperature conditions. Using this method, the percentage of microaggregates in citrate blood incubated for 15 minutes (n=8) and their kinetics 0-3h post phlebotomy (n=8) were determined to establish the effect of potential artefactual platelet activation on microaggregate formation. This was achieved by using ImageStream^X to measure and visualise the localisation of BV421 CD62p (added to the original antibody labelling described above at a 1/100 dilution) on barbell-shaped microaggregates in comparison with single platelets (n=8). Furthermore, the concentration of microaggregates was correlated with the degree of platelet activation (n=5) and studying microaggregate kinetics in the presence of eptifibatide (10µM; an inhibitor of platelet $\alpha_{IIb}\beta_3$ -mediate platelet aggregation; n=5). Also, microaggregate

marginal band morphology was depicted by ImageStream^X by labelling 25 μ L of citrate blood with PBS containing FITC CD61 (1/25) and SiR tubulin (Alexa Fluor 674nm; 4 μ M) or by super high-resolution SIM microscopy of α -tubulin labelled PRP originating from 21°C citrate blood.

Once microaggregates were characterised, preplatelets and barbell platelet were investigated. As true barbell platelets (consisting of a two distal platelet structures interconnected by a thin cytoplasmic bridge) were observed by ImageStream^X and blood films in 37°C citrate blood and no other anticoagulant, preplatelet and barbell discrimination, characterisation and quantitation were further investigated under these conditions.

Preplatelet maturation events were characterised using low resolution widefield Leica (n=6) and super high-resolution SIM microscopy (n=5) using α -tubulin labelling of PRP originating from 37°C citrate blood to describe differences in marginal band morphology during preplatelet/barbell conversion. From these findings, preplatelets and barbell platelets were accurately discriminated using pre-designed ImageStream^X IDEAS analyses (Figure 3.8). The diameter and perimeter of preplatelets were measured by ImageStream^X using FITC CD61 fluorescence morphology of an erode+4 pixel mask and compared to barbell platelets (n=5) to determine barbells originate from large platelets and the size threshold in which platelets can convert into barbells. Using ImageStream^X, preplatelets and barbells were also accurately quantified in healthy donor citrate blood when incubated for 15 minutes at 37°C and compared to IPF measurements (n=12). Finally, 37°C citrate blood was incubated for 3h to determine the kinetics of the changes in concentrations of preplatelets, barbell platelets and IPF

(n=5). Presence of barbells was also determined with or without eptifibatide (10 μ M; n=5).

4.3 Results

4.3.1 Determining Platelet Morphology in 21°C Anticoagulated Human Whole Blood

As aspect ratio determines the difference between the major and minor axis of a cellular image, it is possible to measure the roundness or circularity of an image. Thus, this parameter, in conjunction with area, can be used to estimate how elongated platelets are in given populations. From this plot, 2 regions were drawn: a blue triangle representing compact circular or discoid platelets and a red triangle containing elongated platelet structures (Figure 4.1a). Platelet morphology in EDTA was compact consisting predominantly (>99.5%) of resting discoid/circular platelets (Figure 4.1a (blue triangle gate) & Figure 4.1c). In contrast, hirudin and citrate blood contained a discrete population of elongated platelet microaggregates (~1%) consisting of two distal platelet structures, resembling the barbell shape (situated within the red triangle gate; Figure 4.1a, 4.1b & 4.1c; $p = 0.0235$ & $p = 0.0371$). Additional images of barbell-shaped microaggregates in citrate blood can be found in Appendix Figure 3.2. Microaggregates appeared to be analogous between citrate and hirudin blood however, in hirudin, within this microaggregate population were many larger platelet aggregates consisting of more than 2 platelet structures (Data not shown).

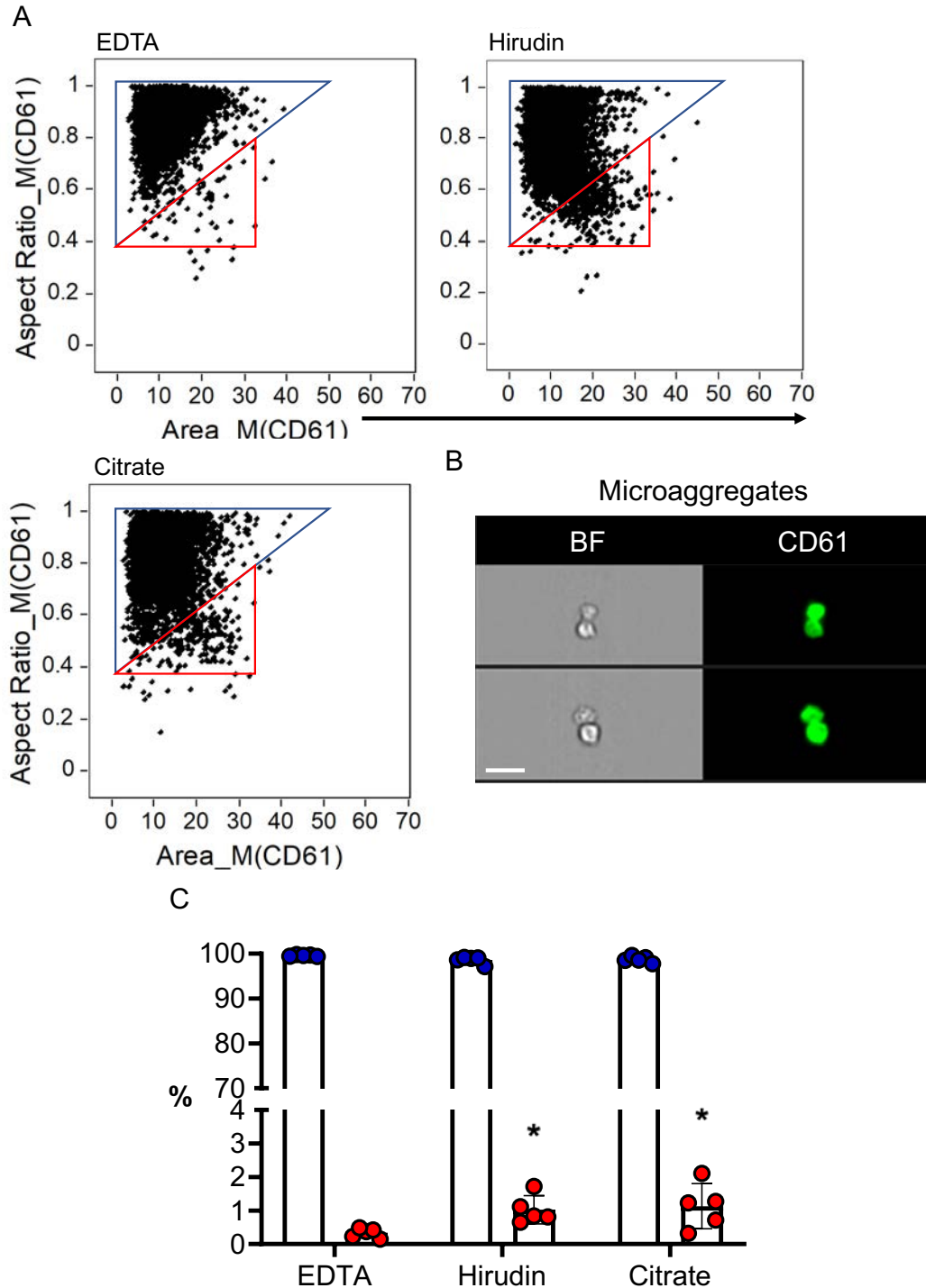


Figure 4.1: Barbell-Shaped Platelet Microaggregates are Present in 21°C Citrate Anticoagulated Whole Blood

Room temperature whole blood from the same donor was anticoagulated in EDTA, hirudin and citrate and platelet morphology was analysed using ImageStream^X. (A) From the platelet cloud described in Figure 3.2, platelet morphology was depicted by aspect ratio (circularity) and area of FITC CD61 fluorescence (n=5). Resting single platelets are situated within the blue triangle gate and elongated platelet structures within the red triangle gate. (B) Examples of barbell-shaped microaggregates in citrate blood situated within the red triangle (scale bar 7 μ m). (C) Fraction of single platelets (blue) and microaggregates (red) in anticoagulated whole blood (n=5; error bars $\pm$ 1 SD). M: morphology; BF: brightfield. (C) Two-way ANOVA with Tukey multiple comparison test; Sig. * <0.05 .

Barbell-shaped microaggregates were also observed in citrate blood films (n=3; Figure 4.2; red arrow). In contrast, platelets in EDTA blood films existed as individual platelets whereas, in hirudin non-barbell shaped aggregates were observed. As a result, the relevance of barbell-shaped microaggregates were further investigated in citrate blood at room temperature.

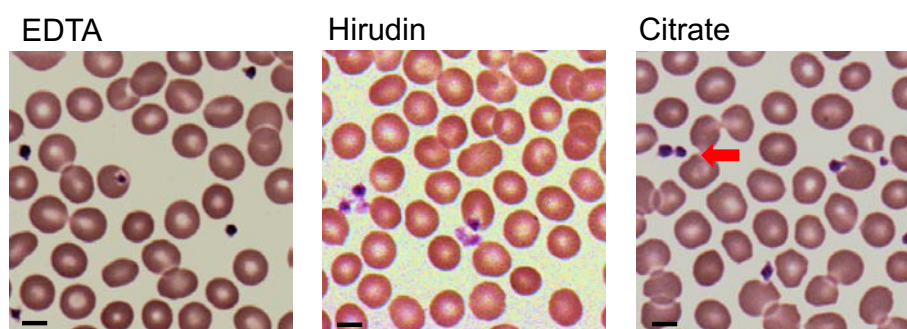


Figure 4.2: Barbell-Shaped Platelet Microaggregates in Blood Films

Blood films originating from EDTA, hirudin and citrated whole blood, stained with modified Giemsa stain (Figure 2.1). Platelets are identified as purple. Red arrows indicate barbell-shaped microaggregates in citrated blood. Images were taken using a Leica DM6000 widefield microscope (n=3; scale bar 5 μ m).

4.3.2 Barbell-Shaped Microaggregates are an Artefact of Platelet Activation

To further characterise barbell-shaped microaggregates in citrate whole blood, an ImageStream^x IDEAS analysis gating strategy was designed for their accurate discrimination and quantification (see Figure 3.11). After a 15-minute incubation at room temperature, the fraction of microaggregates in citrated blood was ~0.75% (n=8; Figure 4.3a).

To determine any potential change in microaggregates time post phlebotomy, citrate blood was incubated at room temperature over time and microaggregates were quantified at 0, 1.5 and 3h time points (n=5). As barbell-shaped microaggregates were

all but absent in EDTA, this anticoagulant was used as a negative control. The percentage of microaggregates in citrate blood significantly increased in a time dependent manner ($t=1.5h$: $p = 0.0029$ & $t=3h$: $p = 0.0198$) whereas, in EDTA such structures remained absent (Figure 4.3b & 4.3c).

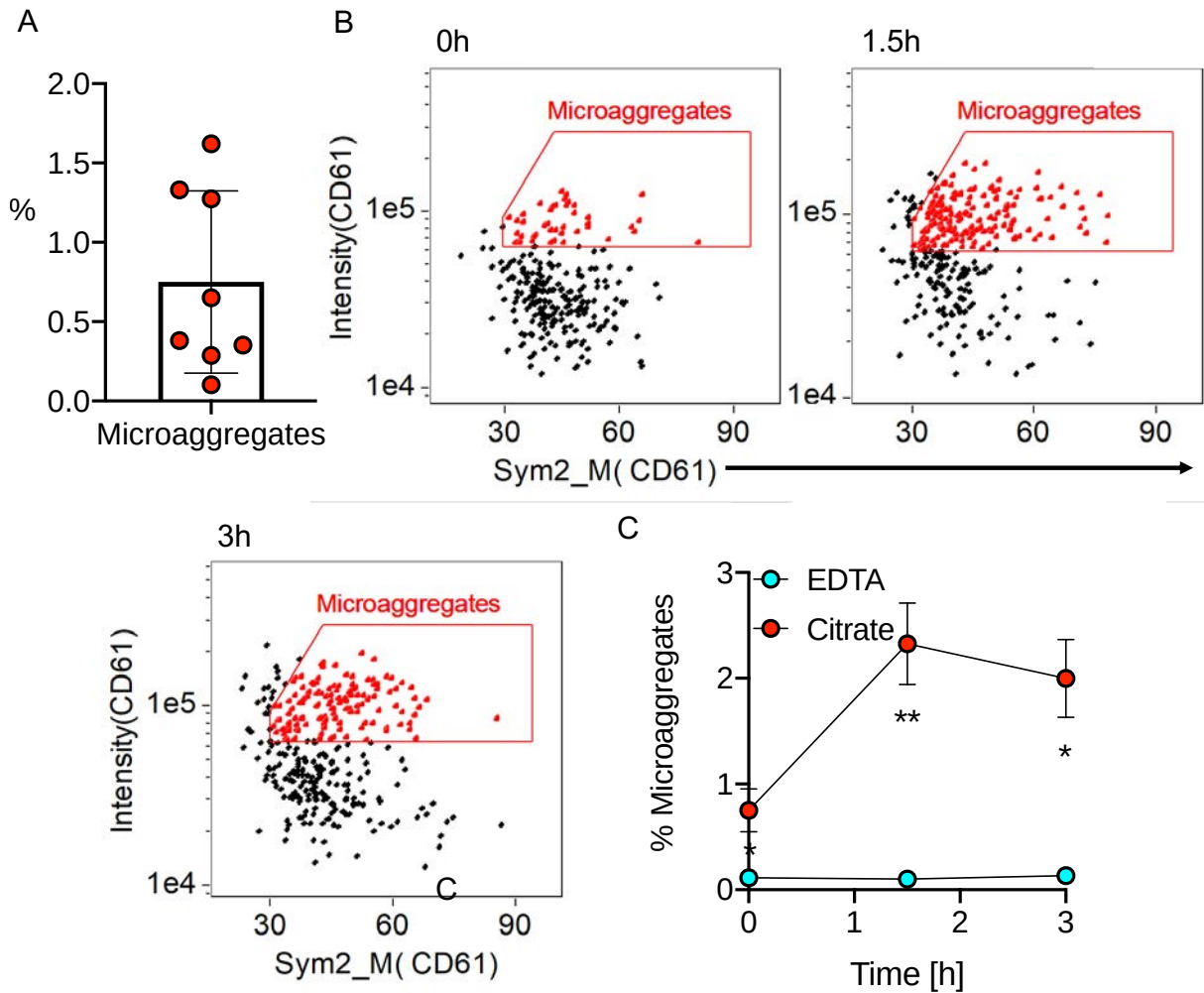


Figure 4.3: Barbell-Shaped Microaggregates in 21°C Citrate Whole Blood Significantly Increase with Time Post Phlebotomy

Whole blood from healthy human donors was anticoagulated in EDTA or citrate and 10,000 FITC CD61/PE CD42b positive platelet images were acquired using the ImageStream^X (Figure 3.2) and CD61 fluorescence morphology was used for all analyses. (A) Barbell-shaped microaggregates were quantified using ImageStream^X (Figure 3.11) in 21°C citrate blood ($n=8$) and (B) change over time was observed at 0, 1.5 and 3h post phlebotomy ($n=5$). (C) microaggregate kinetics were determined in citrate blood and compared to EDTA ($n=5$). Sym2: symmetry2; M: morphology; BF: brightfield. Error bars ± 1 SD. (C) Two-way ANOVA with Bonferroni multiple comparison test; Sig. * <0.05 ; ** <0.01 ; *** <0.001 .

Labelling whole blood with BV421 CD62p showed, platelet microaggregates displayed a significant greater expression of CD62p than discoid platelets (n=8; p = 0.0037; Figure 4.4a). Microaggregates consisted of either one or two activated platelets (Figure 4.4a; see Appendix Figure 4.1 for additional images). Furthermore, platelet activation in citrate and EDTA blood increased with time post phlebotomy (n=5; p = 0.031 & p = 0.0002 respectively; Figure 4.4b) and this positively correlated with the fraction of microaggregates in citrate (r = 0.7639 & p = 0.0009; Figure 4.4c) suggesting barbell-shaped microaggregates are artefactually activated, aggregating platelets and not true barbell platelets.

To determine if microaggregates in room temperature citrate blood are indeed aggregating platelets, healthy donors (n=5) were bled directly into citrate vacutainers® containing eptifibatide (10 μ M) and the concentration of microaggregates/ μ L of blood were quantified by ImageStream^X at 0, 1.5 and 3h time points. Eptifibatide is a potent inhibitor of $\alpha_{IIb}\beta_3$ -mediated platelet aggregation. Platelet aggregometry confirmed eptifibatide (10 μ M) inhibits platelet aggregation when platelets are exposed to ADP (10 μ M; Figure 4.5a). In the presence of eptifibatide (10 μ M), microaggregates were almost eradicated (Figure 4.5b & 4.5c). Also, interrogation of FITC CD61 and BV421 CD62p fluorescence by ImageStream^X shows in the presence of eptifibatide, the few remaining barbell-shaped microaggregates present, lacked the intimate association of those observed in normal citrate blood. Quantification of microaggregates 0-3h post phlebotomy showed microaggregates remained significantly reduced in eptifibatide for 3h (p = <0.0001), even though the total platelet population displayed enhanced CD62p exposure (p = 0.0041; n=8; Figure 4.6a & b).

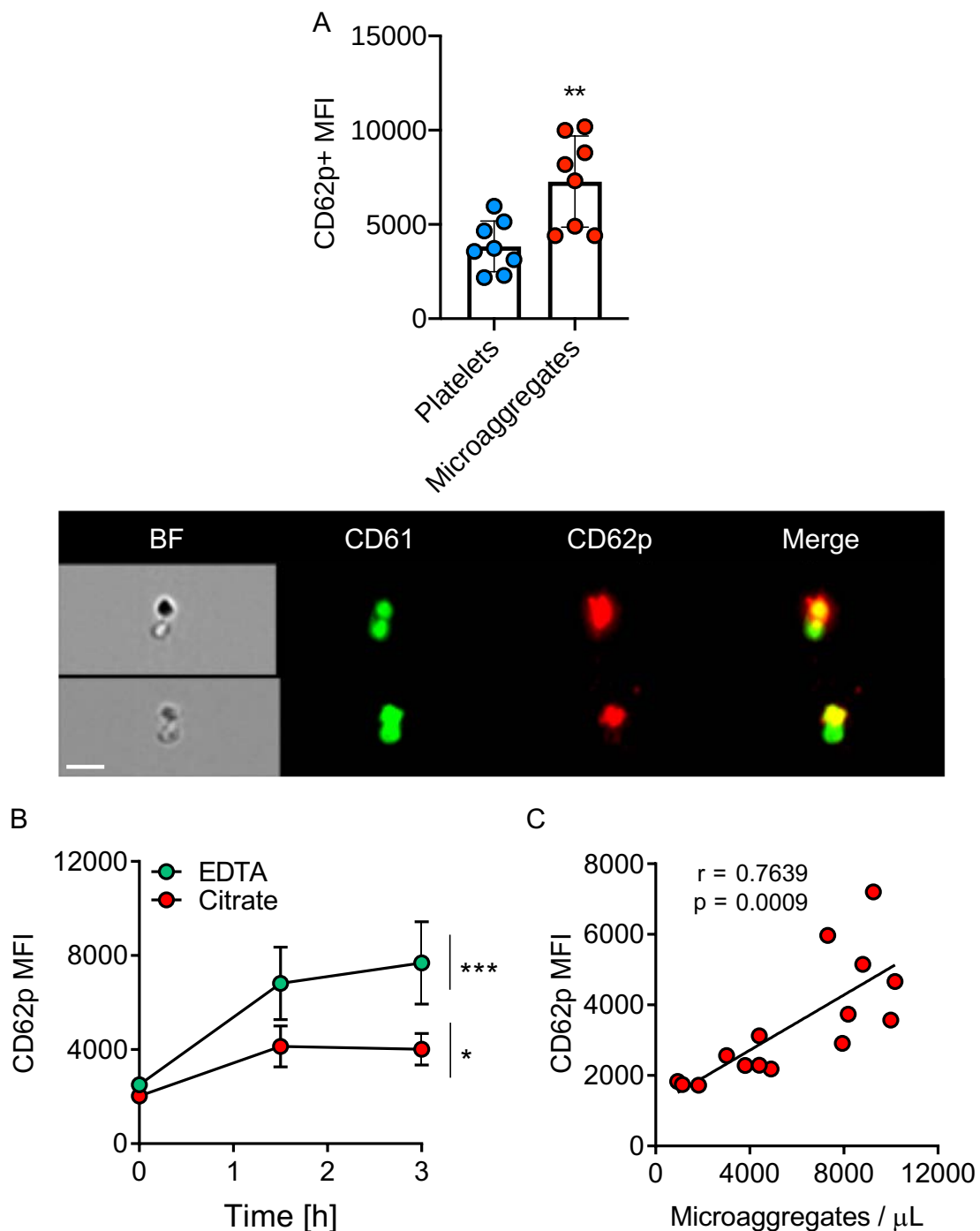


Figure 4.4: Barbell-Shaped Platelet Microaggregates in 21°C Citrate Blood are Caused by Artefactual Platelet Activation

Platelet activation was determined by BV421 CD62p mean fluorescence intensity (MFI). (A) Comparison between total platelet and barbell-shaped microaggregate CD62p exposure in citrate blood (n=5). ImageStream^X microaggregate imagery depicted by brightfield (BF), CD61, CD62p and merged CD61/CD62p fluorescence using x60 lens to show CD62p localisation of activated platelets (n=5; scale bar 7μm). (B) Total platelet activation in citrate and EDTA whole blood 0-3h post phlebotomy (n=5). (C) Relation between the number of microaggregates/μL of citrate blood compared to platelet CD62p MFI. Error bars +/- 1 SD. (A) Paired t test; (B) two-way ANOVA; (C) Pearson's Correlation Coefficient. Sig *<0.05; *** <0.001

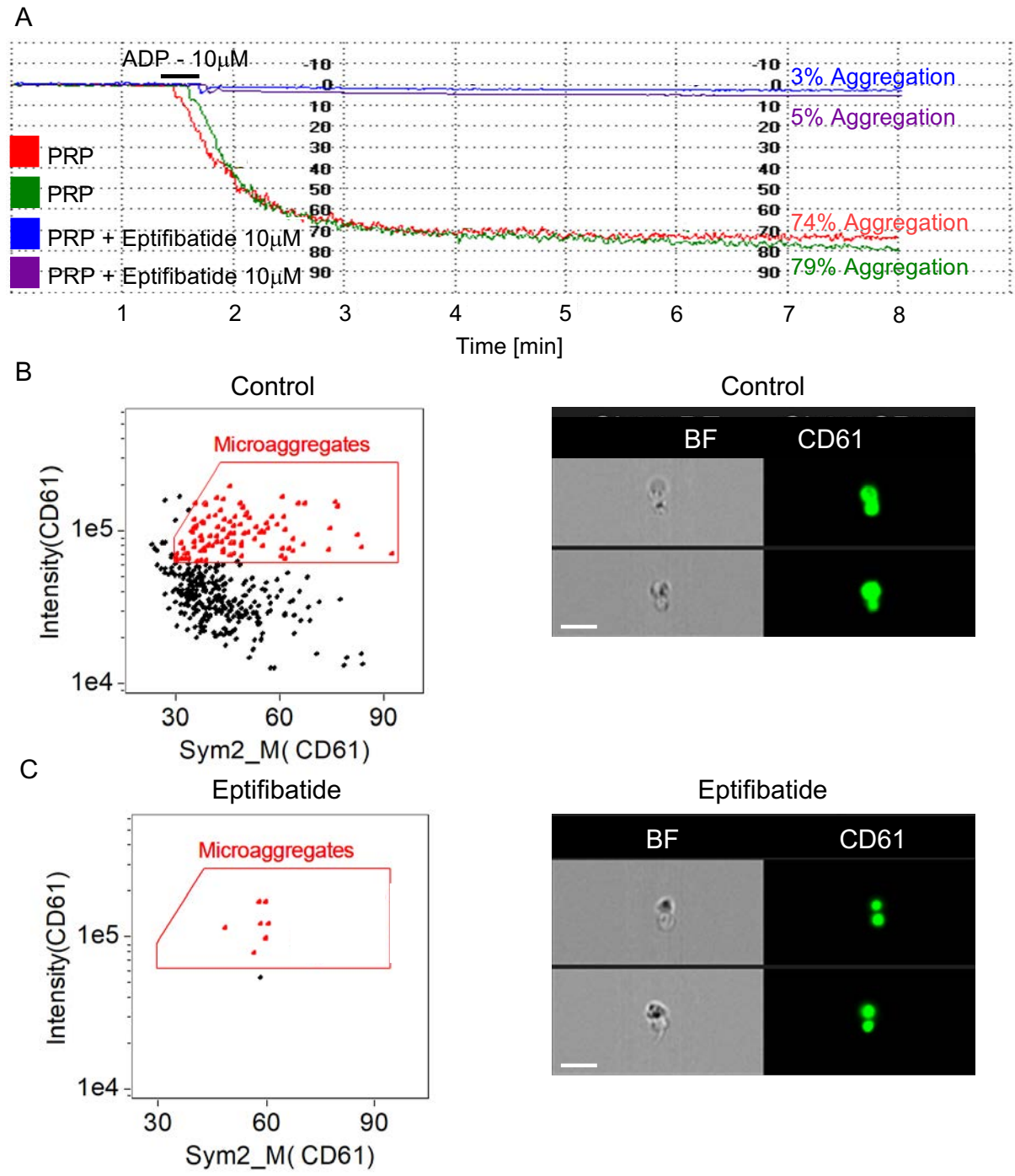


Figure 4.5: The Effect of Eptifibatide on Barbell-Shaped Microaggregates in 21°C Citrate Whole Blood

(A) PRP originating from healthy donor citrate whole blood with or without eptifibatide (10 μ M) was exposed to 10 μ M ADP (Adenosine Diphosphate) and platelet aggregation was measured using the PAP-8E aggregometer (n=3; representative of a single experiment measured in duplicate). (B-C) Citrate blood was exposed to eptifibatide (10 μ M) immediately following venepuncture and 10,000 FITC CD61/PE CD42b positive platelet images were acquired using the ImageStream^x to determine the effect on microaggregate formation. Microaggregates were measured from CD61 fluorescence morphology (see Figure 3.11). Images of microaggregates were depicted by brightfield (BF) and CD61 fluorescence (x60 lens, scale bar 7 μ m).

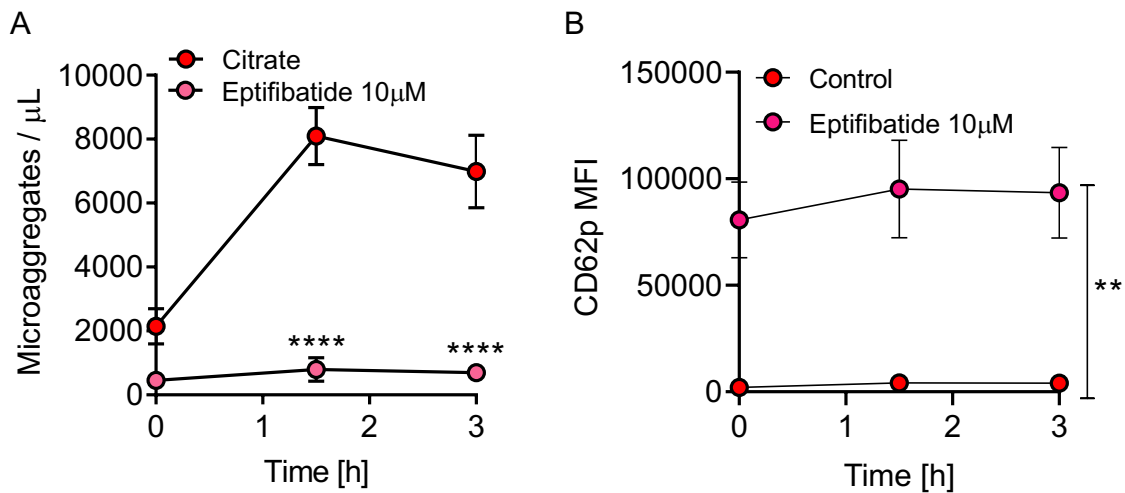


Figure 4.6: The Effect Eptifibatide on the Kinetics of Barbell-Shaped Microaggregates in Citrate Blood

Citrate blood was exposed to eptifibatide (10 μ M) and labelled with BV421 CD62p, FITC CD61 & PE CD42b. 10,000 CD61/CD42b positive platelet images were acquired using the ImageStream^X. Microaggregate quantification was based on CD61 fluorescence morphology (see Figure 3.11). (A) Microaggregates were quantified immediately following phlebotomy. At 0, 1.5 and 3h post phlebotomy (n=5). (B) Total platelet activation (determine by CD62p mean fluorescence intensity: MFI) in citrate whole blood with or without eptifibatide (10 μ M) 0-3h post phlebotomy. Error bars +/- 1 SD. (A-B) two-way ANOVA with Bonferroni multiple comparison test. Sig. ** <0.01; **** <0.0001.

4.3.3 Barbell-Shaped Microaggregates Consist of Two Individual Platelets with Marginal Bands

To definitively prove barbell-shaped microaggregates are comprised of two individual aggregating platelets, marginal band morphology was depicted by tubulin labelling. If microaggregates are indeed two platelets they will contain two separate marginal bands and not the continuous figure-of-eight morphology observed in barbell platelets [380, 382]. To identify microaggregate marginal band morphology, platelets in citrate blood or PRP were labelled directly with anti-human FITC CD61 and 4 μ M AF674nm SiR tubulin or indirectly with goat anti-mouse α -tubulin (AF488 labelling) and analysed directly in blood using the ImageStream^X (x60 lens; n=5) or super high-resolution SIM microscopy (x100 lens; n=6) respectively.

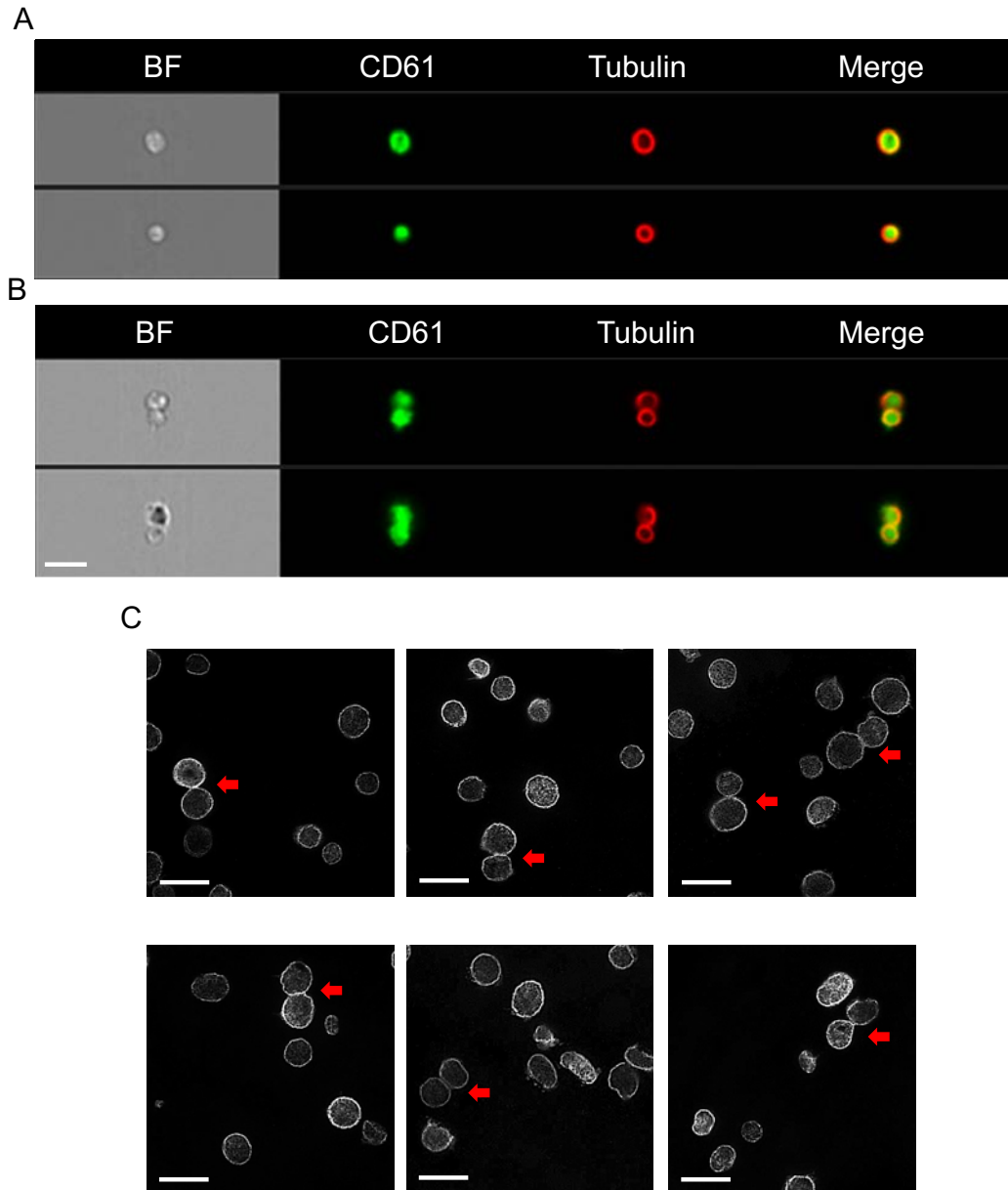


Figure 4.7: Barbell-Shaped Platelet Microaggregates Consist of Two Individual Platelets with Separate Marginal Bands

Citrate whole blood was probed with anti-human FITC CD61, PE CD42b and Alexa Fluor (AF) 674 SiR tubulin (4 μ M) for 30 minutes at 21°C and 10,000 CD61/CD42b platelet images were acquired by the ImageStream^x and barbell-shaped microaggregate were discriminated using CD61 fluorescence (Figure 3.11). (A) ImageStream^x image gallery displaying (A) resting platelet and (B) barbell-shaped microaggregate morphology. Images are depicted by brightfield (BF), FITC CD61, AF647 SiR tubulin and merged CD61/SiR tubulin (Merge) images (x60 lens, scale bar 7 μ m). (C) PRP originating from citrate whole blood was indirectly labelled with goat anti-mouse α -tubulin, secondary labelled with rabbit anti-goat AF488 (see General Methods 2.13) and imaged using super high-resolution SIM microscopy. Red arrows indicate barbell-shaped microaggregates consisting of two separate marginal bands (x100 lens; n=6; scale bar 5 μ m).

Using ImageStream^X, SiR tubulin labelling demonstrated that resting platelets in citrate blood contained a normal marginal band morphology (Figure 4.7a). In contrast, barbell-shaped microaggregates consisted of two distal marginal bands (Figure 4.7b) From this analysis, the majority of microaggregate images obviously contained two individual marginal bands however, due to limitations in resolution, it was unclear with some images.

Alternatively, using super high-resolution SIM microscopy, α -tubulin labelled PRP demonstrated barbell-shaped microaggregates consisted of two individual marginal bands (n=6; Figure 4.7c). No figure-of-eight marginal band structures were observed using either ImageStream^X or microscopy under these conditions suggesting preplatelet barbell formation is highly dependent on temperature. In conclusion, barbell-shaped microaggregates in room temperature citrate blood are likely to be an artefact of ex-vivo platelet activation and can exclude from further barbell platelet analyses using CD62p fluorescence.

4.3.4 Identifying Preplatelet Maturation Events in 37°C Anticoagulated Whole Blood

As for room temperature anticoagulated blood analyses, whole blood from a single healthy human donor (n=5) was anticoagulated in EDTA, hirudin and citrate and platelet morphology was determined by ImageStream^X and blood film imaging.

For ImageStream^X analyses, anticoagulated blood was immediately labelled with anti-human FITC CD61, PE CD42b and BV421 CD62p at 37°C for 15 minutes (preventing the cooling of blood), fixed in preheated 2% formalin and platelet morphology was

determined by aspect ratio and area of FITC CD61 fluorescence morphology (Figure 4.8a).

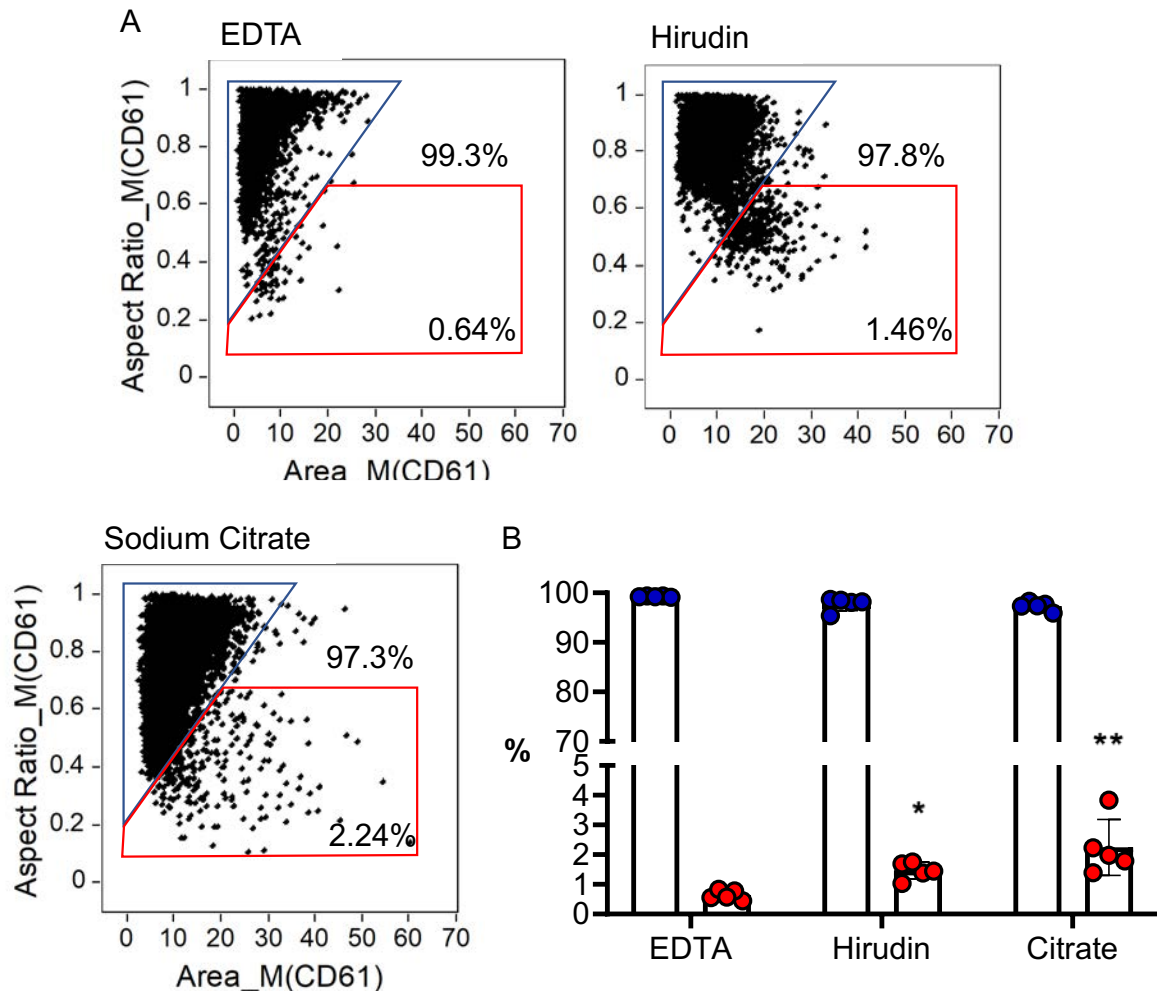


Figure 4.8: Barbell Platelets are Present in 37°C Citrate Whole Blood

All experiments were conducted at 37°C. Whole blood from the same donor was anticoagulated in EDTA, hirudin and citrate and platelet morphology was analysed using ImageStream^X. (A) 10,000 FITC CD61 / PE CD42b positive platelet images were acquired using ImageStream^X (Figure 3.2) and platelet morphology was depicted by aspect ratio (circularity) and area of CD61 fluorescence morphology (n=5). From this plot, single platelets are situated within the blue triangle region and elongated platelets within the red triangle region. (B) Fraction of single platelets (blue) and total elongated platelets (red) in anticoagulated whole blood (n=5). Error bars +/- 1 SD. M: morphology. B) Two-way ANOVA with Tukey multiple comparisons test; Sig. *<0.05; **<0.01.

Platelets in EDTA remained discoid (blue region; 99.3%) with a small fraction (red region; 0.64%) of elongated platelet structures which included barbell-shaped microaggregates observed at room temperature (Figure 4.8a & 4.8b and Figure 4.1a & 4.1b; see Appendix Figure 3.2 for additional microaggregate images). Platelets in

37°C hirudin blood also displayed similar morphological characteristics to that observed at room temperature with 97.8% single platelets and 1.46% elongated with the latter containing both barbell-shaped microaggregates and larger aggregates (Figure 4.8a & 4.8b and Figure 4.1a & 4.1b). In contrast, in 37°C citrate blood, platelets became more elongated (2.24%; $p = 0.0025$). Interestingly, within this region, CD62p negative barbell structures consisting of two distal platelet structures interconnected by a thin cytoplasmic bridge (a key characteristic of barbell platelets) were present (Figure 4.9; see Appendix Figure 4.2 for additional images of barbells). However, also within this region were CD62p positive barbell-shaped microaggregates (examples of CD62p positive microaggregate images are found in Appendix Figure 4.1).

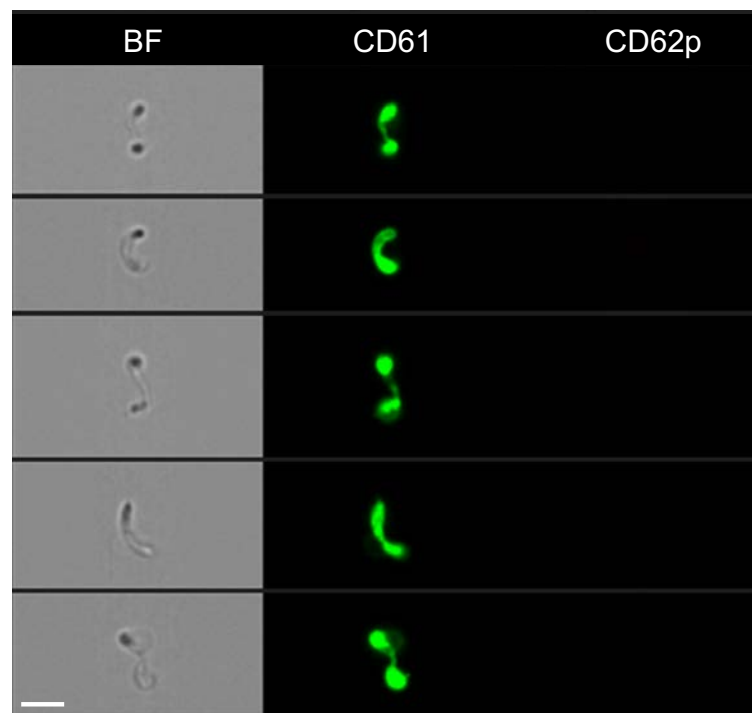


Figure 4.9: Barbell Platelets in 37°C Citrate Blood

ImageStream^X image gallery showing examples of barbell platelet morphology – depicted by brightfield (BF), FITC CD61 and BV421 CD62p fluorescence; x60 lens, scale bar 7µm).

Barbell platelets were also present in 37°C citrate blood films and undetectable in EDTA and hirudin (n=3; Figure 4.10). However, within hirudin blood, barbell-shaped platelet microaggregates and larger aggregates were present. As a result, all ongoing preplatelet and barbell platelet analyses were conducted using platelets from 37°C citrate blood.

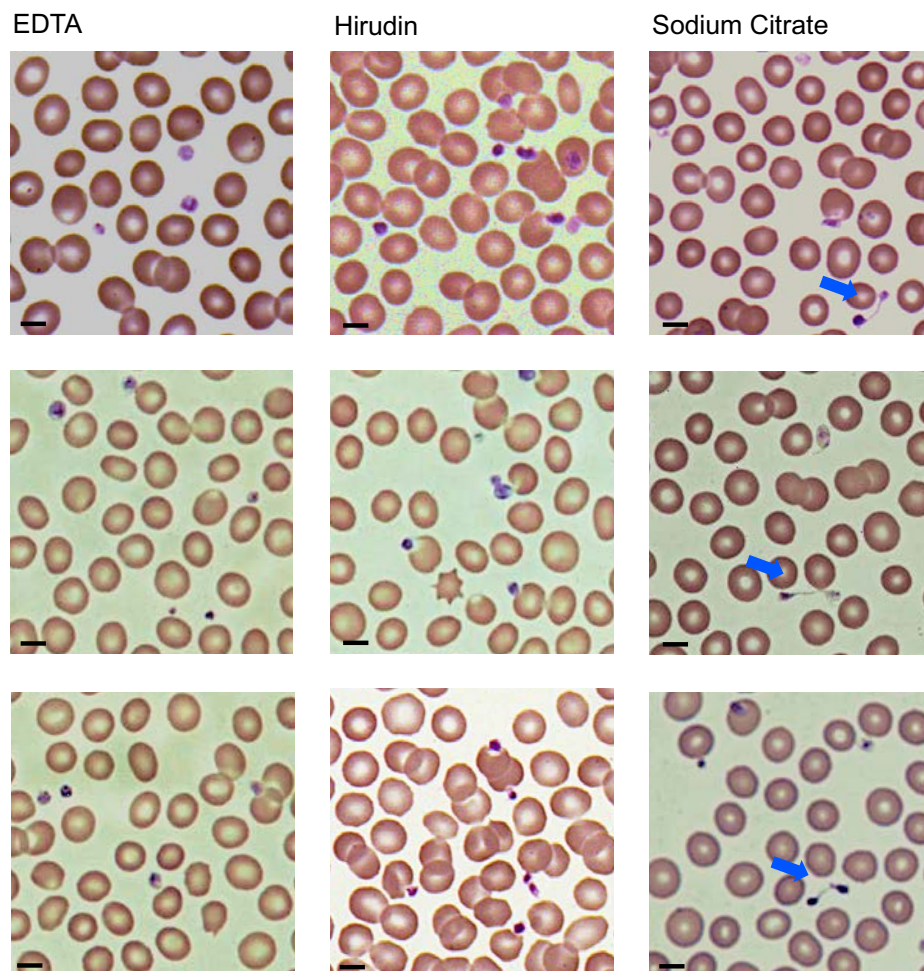


Figure 4.10: Barbell Platelets are Present in 37°C Citrate Blood Films

Whole blood from a single donor (n=3) was anticoagulated in EDTA, hirudin and citrate. To prevent cooling, blood was immediately placed in a water bath (37°C) for immediate blood film analysis. Blood films were fixed and stained with Giemsa as above. Blood films were imaged by widefield using the DM6000 Leica microscope (x60 lens). Blue arrows indicate barbell platelets. Scale bar 5µm.

4.3.5 Characterising Preplatelet Maturation in 37°C Citrated Human Whole Blood

To determine the morphology of platelet marginal bands in 37°C citrate blood, PRP was generated from citrate blood under strict 37°C temperature control (totalling ~15 minutes preparation time), indirectly labelled with goat anti-mouse α -tubulin and imaged by widefield microscopy using the DM600 Leica (x60 lens; n=6).

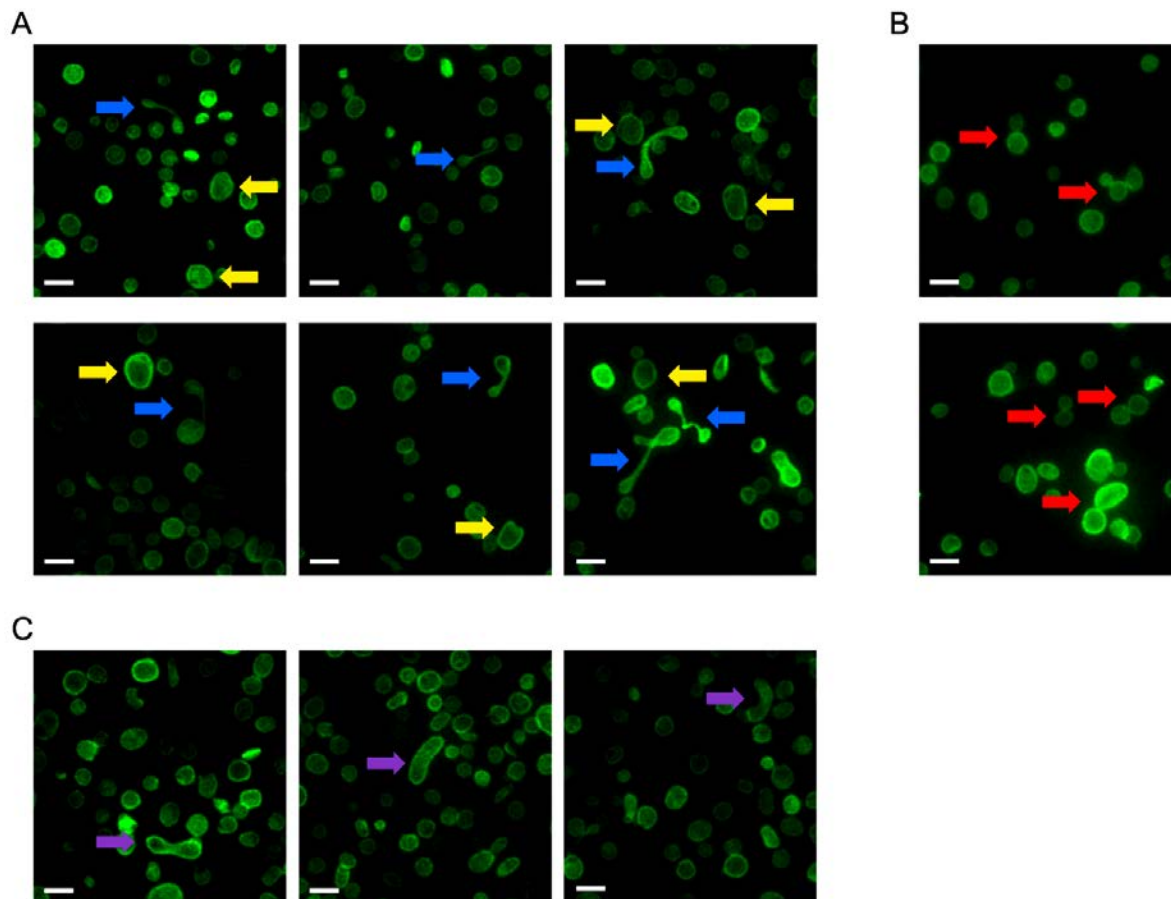


Figure 4.11: Characterising Marginal Band Morphology During Preplatelet Barbell Conversion

Human PRP originating from citrate whole blood was prepared immediately following phlebotomy under strict 37°C temperature control and probed with a goat anti-mouse α -tubulin monoclonal antibody, secondary labelled with a rabbit anti-goat 488 and imaged by widefield using the DM6000 Leica microscope (x60 lens). (A) Barbell platelets are shown by blue arrows and preplatelets by yellow arrows. (B) barbell-shaped microaggregates shown by red arrows. (C) Elongated oval-shaped preplatelets transforming into barbell platelets are shown by purple arrows. (n=6; scale bar 5 μ m). Each demonstrative region of interest is representative of a single experiment.

Barbell platelets consisted of two distal platelet structures interconnected by a thin cytoplasmic bridge or shaft positive for tubulin (Figure 4.11a; blue arrows). Also, present, were preplatelets $>3\mu\text{m}$ in diameter (yellow arrows). Barbell-shaped microaggregates were also observed (Figure 4.11b; red arrows). However, as previously reported these structures are comprised of two independent marginal bands (Figure 4.7). In addition to barbell platelets, circular preplatelets and microaggregates, were elongated preplatelets lacking a thin cytoplasmic bridge (Figure 4.11c; purple arrows). These structures appear to be an intermediate stage of preplatelet barbell conversion.

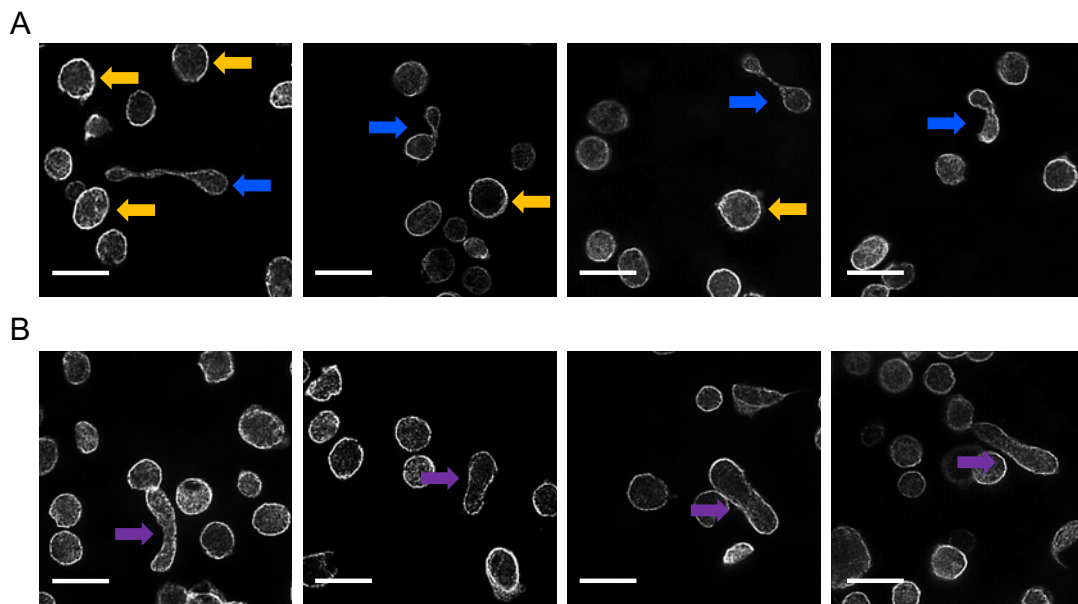


Figure 4.12: Super High-Resolution Imaging of Preplatelet Barbell Conversion

Human PRP originating from citrate whole blood was prepared and labelled as in Figure 4.11 and imaged by super high-resolution SIM microscopy (x100 lens; n=5). (A) Circular preplatelets ($>3\mu\text{m}$ in diameter) are shown by yellow arrows and barbell platelets shown by blue arrows. (B) intermediate preplatelet elongates are shown by purple arrows. Scale bars $5\mu\text{m}$.

To characterise these findings in greater detail, α -tubulin labelled PRP from 37°C citrate blood was imaged using super high-resolution SIM microscopy (x100 magnification; n=3). Preplatelets contained a normal circular marginal band $>3\mu\text{m}$ in diameter (Figure 4.12a; yellow arrows). In contrast, barbell platelets consisted of a continuous figure-of-eight marginal band, where microtubules extended through the narrow cytoplasmic bridge or shaft and looped back at the distal ends to form two teardrop-shaped structures analogous to that observed in MK proplatelet formation [163]. Alternatively, intermediate elongated preplatelets were characterised by an oval-shaped marginal band (Figure 4.12b; purple arrows).

To accurately discriminate and further characterise preplatelets and barbells, they were distinguished in whole blood using ImageStream^X (see chapter 3 for complete method development and specifically Figure 3.8 for final gating strategy). Based on previous mathematical modelling it is predicted barbells originate from preplatelets 3-10 μm in diameter [384]. Using this prediction, the perimeter of preplatelets $>3\mu\text{m}$ in diameter was measured by ImageStream^X (Figure 4.13a; n=7) and compared to that of barbell platelets. The mean preplatelet diameter was $\sim 3.4\mu\text{m}$ whereas the perimeter range was between 13 and 24 μm (Figure 4.13b). In contrast, the perimeter of barbell platelets was 13 to 42 μm . This confirmed the smallest barbell perimeter (13 μm) equated to that of preplatelets suggesting their diameter must be $\geq 3\mu\text{m}$ in diameter to convert into barbell platelets. The mean perimeter of preplatelets and barbells significantly differed (~ 15 and $\sim 22\mu\text{m}$ respectively; $p = <0.0001$) suggesting circulating preplatelets with a perimeter $>22\mu\text{m}$ convert into barbells more rapidly than smaller cells (Figure 4.13c)

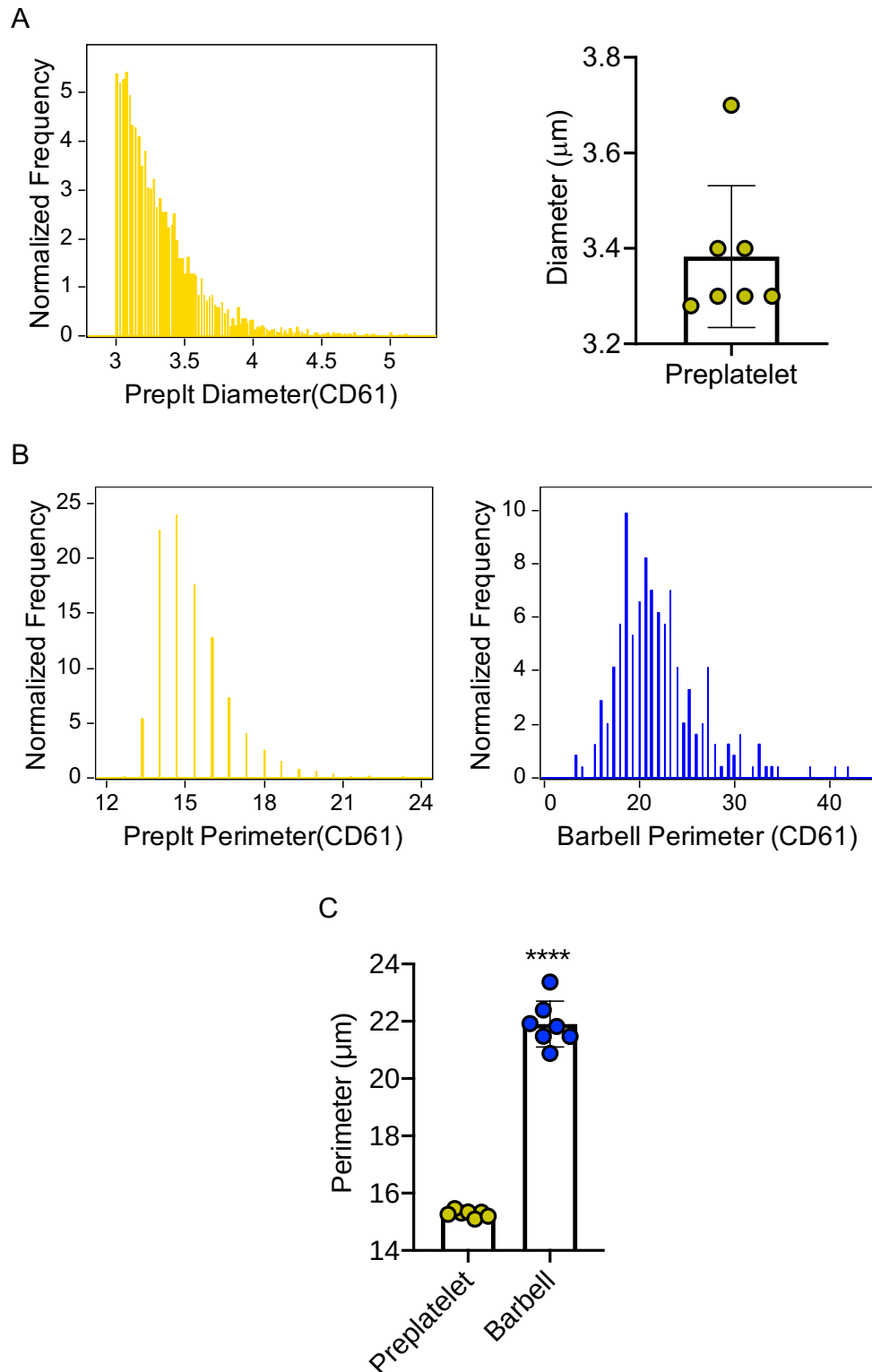


Figure 4.13: Morphometric Quantitation of Preplatelets and Barbell Platelets in 37°C Citrate Whole Blood

Using the ImageStream^X, preplatelets and barbell platelets were discriminated (Figure 3.8) in citrate whole blood incubated at 37°C for 15 minutes (n=7). Diameter and perimeter measurements were calculated using an erode+4 pixel mask of CD61 fluorescence. (A) Preplatelet diameter range and mean. (B) Preplatelets (Preplt) and barbell perimeter ranges. (C) comparison between mean perimeter of preplatelets and barbell platelets. Error bars +/- 1 SD. (C) paired t test. Sig. ****<0.0001.

4.3.6 IFC Quantitation of Preplatelets and Barbell Platelets in Normal Blood

Using the same ImageStream^x method as refined above and in Figure 3.2 & 3.8, preplatelets and barbell platelets (additional images of barbells and preplatelets in healthy control citrate blood can be found in Appendix Figure 4.2 & 4.3 respectively) were quantified in normal blood and compared to IPF measurements determined by the XN1000 analyser (Sysmex). The percentage barbell platelets in citrate whole blood were 0.5%, significantly less than preplatelets and IPF ($p = 0.0014$ & $p = <0.0001$ respectively; Figure 4.14a). Alternatively, the fraction of preplatelets (3.3%) was comparable to IPF (3.4%; $p = 0.9656$). The percentage of preplatelets and barbell platelets and combined fractions positively correlated with the IPF% measured in EDTA whole blood ($r = 0.6191$ / $p = 0.0422$; $r = 0.7308$ / $p = 0.0069$; $r =$ respectively; Figure 4.14b & c). Importantly, combined fractions of preplatelets and barbells from a single individual equated to the IPF in EDTA blood (Figure 4.14e). Interestingly, in citrate blood, there was an ~0.5% decrease in IPF. This decrease was equivalent to the fraction of barbell platelets.

To determine the potential kinetics of changes in preplatelets, barbell platelets and IPF with time post phlebotomy, citrate blood was incubated for 3h at 37°C and measurements were taken at 0, 1.5 and 3h. Although all fractions slightly decreased with time, this trend was not significant (Figure 4.15a). Compared to room temperature, platelet CD62p expression 0-3h at 37°C was significantly reduced ($p = <0.0001$; Figure 4.15b) and the concentration of barbells 0-3h at 37°C did not correlate with CD62p expression (Figure 4.15c).

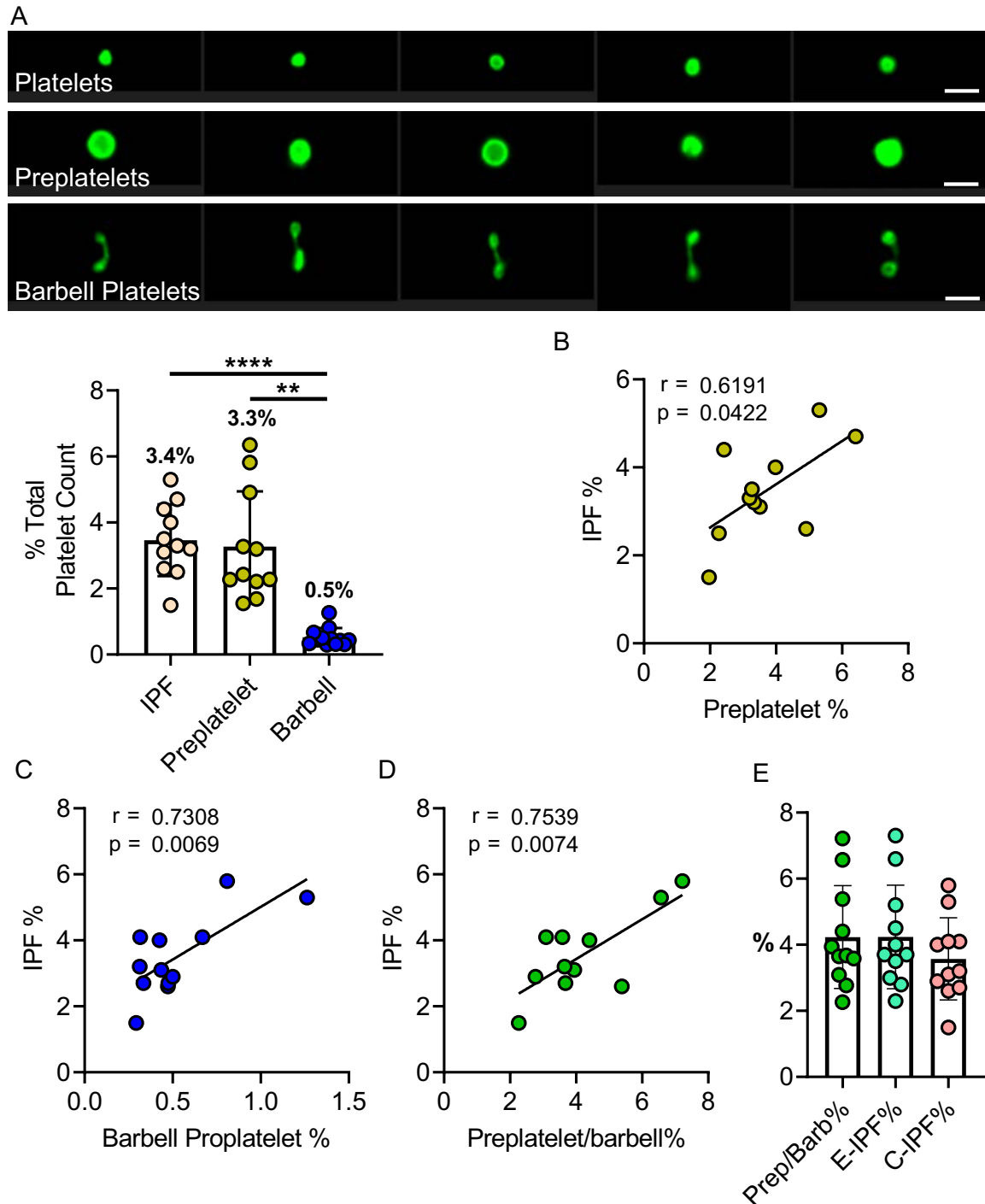


Figure 4.14: ImageStream^X Quantitation of Preplatelets and Barbell Platelets in Normal Blood

Healthy control citrate blood was incubated for 15 minutes at 37°C and labelled with BV421 CD62p, FITC CD61 & PE CD42b. 10,000 CD61/CD42b positive platelet events were imaged by ImageStream^X (n=12). (A) platelets, preplatelets and barbell platelet were measured by ImageStream^X (Figure 3.8). IPF was measured by the XN1000 (Sysmex). Representative images of platelets, preplatelets and barbells depicted by CD61 fluorescence (x60 lens, scale bar 7µm). Correlations between the percentage (B) preplatelets, (C) barbell platelets or (D) combined fractions with IPF% (n=12). Error bars +/- 1 SD. (B-D) Pearson's Correlation Coefficient; (C) one way ANOVA with Tukey multiple comparison test. *<0.05; **<0.01; *** <0.001, ****<0.0001. E-IPF%: % IPF in EDTA; C-IPF: % IPF in citrate.

Finally, barbell platelets were completely absent in 37°C citrate blood pre-incubated with eptifibatide (10 μ M; Figure 4.15d). Eptifibatide binds to the Arg-Gly-Asp binding motif of the β 3 subunit, the same subunit which the CD61 antibody binds. Thus, if eptifibatide interferes with the CD61 binding the signal could be lost along the barbell shaft resulting in two singular platelet objects instead of an elaborately interconnected barbell structure. To answer this, CD61 MFI of platelets with or without eptifibatide was compared. No difference was reported (Figure 4.15e), suggesting the absence of barbells was not due to the loss of the CD61 fluorescent signal along the barbell cytoplasmic bridge. Furthermore, manual interrogation of brightfield imaging confirmed no barbells were present (data not shown).

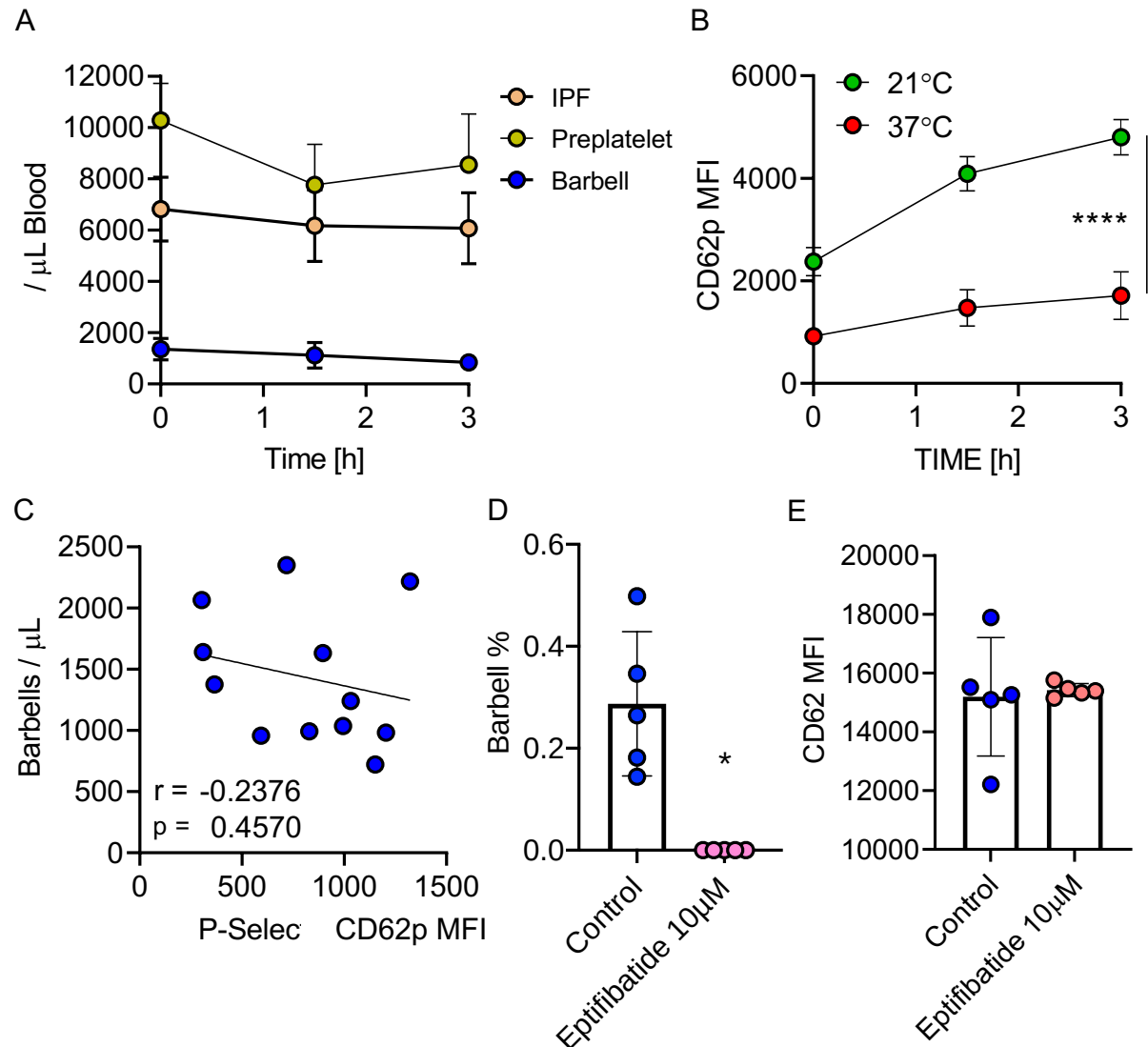


Figure 4.15: Preplatelet and Barbell Platelet Kinetics in Citrate Blood

(A) citrate whole blood was incubated for 3h at 37°C and labelled with BV421 CD62p, FITC CD61 & PE CD42b. 10,000 CD61/CD42b positive platelet images (Figure 3.2) were acquired by ImageStream^x at 0, 1.5 & 3h. The change in the concentration of preplatelets and barbell platelets (Figure 3.8) was measured by ImageStream^x and IPF by XN1000 (n=5). (B) Total platelet CD62p expression was measured in 21 & 37°C whole blood 0-3h post phlebotomy. (C) Correlation between concentration of barbells in citrate blood compared to total platelet CD62p expression. (D) Barbell platelets and (E) total platelet CD61 MFI measured by ImageStream^x (Figure 3.8) in 37°C citrate whole blood with or without eptifibatide 10μM. Error bars +/- 1 SD. (A & B) two-way ANOVA with Bonferroni multiple comparison test. (C) Pearson's Correlation Coefficient; (D & E) paired t test. Sig. *<0.05; ****<0.0001. MFI: mean fluorescence intensity.

4.4 Discussion

By utilising ImageStream^X and blood film analysis with low- and high-resolution imaging of tubulin-labelled PRP we have shown the presence of preplatelet maturation events in healthy donor blood was highly dependent on citrate anticoagulation and 37°C temperature. Furthermore, quantitation of barbell platelets relates to IPF measurements suggesting, they are immature RP and may represent the rate of platelet production under normal resting conditions.

4.4.1 Barbell-Shaped Platelet Microaggregates are an Artefact of Platelet Activation

Previous data within our group (Metcalf, 2016, unpublished observations) showed it was possible to rapidly detect barbell-shaped platelet ‘doublets’ in citrate blood under room temperature conditions using ImageStream^X. Although these structures were barbell-shaped (consisting of two distal platelet structures), they were not only more compact compared to the elaborately elongated figure-of-eight barbell platelets previously observed by others [380, 382] but also lacked a cytoplasmic bridge interconnecting the platelet structures. Furthermore, as Metcalf also observed that barbell-shaped platelet “doublets” were completely absent in EDTA anticoagulated blood in agreement with a previous study by (Thon *et al*, 2012) [384], it was hypothesised such structures were likely to be barbell platelets.

Considering this, we used ImageStream^X to depict platelet morphology in EDTA, hirudin and citrate blood from a single healthy donor (n=8). In agreement with Metcalf (unpublished; 2016) we report there was a discrete population of barbell-shaped platelet doublet structures (~0.75%) within room temperature citrate blood but not in

EDTA. However closer examination suggested that these structures could be “microaggregates” (Figure 4.1, 4.2 & 4.3) consisting of two intimately joined CD61 positive but distal platelet structures lacking a thin interconnecting cytoplasmic bridge. These microaggregates were also in hirudin blood along with many other larger aggregates consisting of more than two platelets. In order to further characterise microaggregates in citrate blood, an ImageStream[×] IDEAS gating strategy was designed for their accurate discrimination (Figure 3.11). Labelling with CD62p demonstrated that they not only consisted of one or two activated platelets but that their numbers increased with time and correlated with the degree of platelet activation (Figure 4.3, 4.4 & 4.5). They were also significantly reduced when inhibiting $\alpha_{IIb}\beta_3$ -mediated aggregation with eptifibatide (Figure 4.6). Although CD62p expression also increased in the presence of eptifibatide, it is known that paradoxical increases in CD62p expression can occur in the presence of $\alpha_{IIb}\beta_3$ inhibitors despite inhibiting fibrinogen mediated platelet aggregation [423].

Furthermore, ImageStream[×] imaging of SiR tubulin labelled citrate blood in conjunction with super high-resolution imaging of α -tubulin labelled PRP (originating from citrate blood), confirmed barbell-shaped microaggregates consist of two adjacent platelet structures containing individual and intact marginal bands (Figure 4.7). Thus, it is highly likely that these microaggregates were an artefact of ex-vivo platelet activation during or following phlebotomy.

It is well reported that non-physiological temperatures such as 4°C can activate platelets and induce spherocytosis and rises in intracellular calcium concentrations [120, 424-426]. Actin filaments are reorganised, and microtubules depolymerise causing loss of the circumferential marginal band and thus the observed shape-

change. Such adverse effects are reduced in platelets stored at room temperature (equivalent to hypothermic conditions) [427]. As a result, these conditions are used for storing platelets for transfusion. However, platelets prepared and stored for transfusion develop platelet storage lesions causing a multitude of adverse outcomes mainly affecting metabolism, function and morphology [428-430].

Despite this, others have reported changes in platelet morphology when incubated at room temperature [426, 431]. This is thought to be a result of artefactual activation. Maurer-Spurej *et al* (2001) used phase contrast microscopy and scanning and transmission electron micrographs of citrated PRP incubated for 10 minutes at 20°C, 37°C and 42°C, to show platelets at 20°C had altered morphology with pseudopodia formation. Also, ultrastructurally, marginal bands were positioned proximally or absent [432]. In contrast, from the same study, platelets at 37°C and 42°C displayed normal morphology and ultrastructure containing a normal peripherally positioned marginal band. Interestingly, platelet morphology of 20°C platelets returned to normal when incubated at 37°C for 15 minutes. Furthermore, flow cytometry of PAC-1 binding showed citrate platelets were more activated than 37°C and 42°C conditions.

We also observed significant increases in platelet activation (determined by CD62p expression; $p < 0.05$; Figure 4.4c) in room temperature citrate blood platelets up to 3h post phlebotomy which correlated ($p = < 0.0001$) with increases in the percentage of microaggregates (Figure 4.4d). In contrast, platelet CD62p expression in 37°C citrate blood was significantly reduced than that observed at 21°C (Figure 4.4d & 4.15b).

CD62p positive barbell-shaped microaggregates were also observed by IFC in 37°C citrate blood, however, unlike those observed at room temperature the population was no longer discrete (Figure 4.8). Situated amongst these microaggregates were large

numbers of elongated barbell platelets with similar morphological attributes determined by aspect ratio and area of CD61 (Figure 4.9). As these barbell-shaped platelets were CD62p negative, it was therefore completely possible to eliminate artefactual microaggregate barbell-shaped contaminants from future barbell analyses (Figure 3.12).

4.4.2 Preservation of Preplatelet Maturation Events in Whole Blood is Dependent on Anticoagulant and Temperature.

As no true barbell platelets were present in anticoagulated blood maintained at room temperature, we analysed platelet morphology in blood maintained at 37°C. To ensure whole blood remained at 37°C, blood was immediately taken to the laboratory following venepuncture and all antibody labelling and fixation steps were performed with preheated reagents. ImageStream^X and blood films showed barbell platelets were only present in citrate blood and completely absent in both EDTA and Hirudin (Figure 4.9 & 4.10).

The three anticoagulants used in this study were selected as they differ in their anticoagulant properties and mechanisms. EDTA and citrate are calcium chelators. Citrate is a weak chelator of extracellular calcium and as its effects are reversible it is universally used in platelet function tests such as aggregometry [433]. EDTA however, is a divalent cation chelator which irreversibly sequesters free calcium and spheres platelets.

EDTA induces platelet spherizing when incubated for 30 minutes at 37°C. Under these conditions, platelet ultrastructural modifications have been observed by electron microscopy, particularly affecting the plasma membrane and the lining of the OCS

[331]. The OCS displayed abnormal characteristics with distended channels and such expansions caused the collapse of other channels which become narrower and extended through the cell body. As a result, it is highly likely EDTA caused barbell platelets to become spheroid and convert back into preplatelets.

EDTA-induced spherocytosis has also been reported in circulating proplatelets observed in blood from the venous and aortic circulation of rats treated with anti-platelet serum (APS) [381]. In agreement with this, when investigating the microtubule dynamics of preplatelet/barbell conversion in normal blood samples, Thon and colleagues (2012) observed rapid EDTA-induced spherocytosis of preplatelets [384]. Like our study, blood from healthy donors was anticoagulated with EDTA, citrate, and heparin (instead of Hirudin). Showing key comparisons with our study (Figure 4.10) blood film analysis showed barbell platelets were observed in citrate and were absent in EDTA and heparin. In Thon's study, qualitative evidence from β 1-tubulin labelling of PRP originating from the anticoagulated blood samples suggested platelets were smaller in EDTA and heparin consisting of a constricted marginal band microtubule coiling. Thus, platelet marginal band diameter and thickness are critical factors which permit preplatelet/barbell conversion. Paradoxically, platelets in EDTA have been shown to induce platelet swelling in a time dependent manner [332, 434]. To explain such discrepancies, Thon and colleagues (2012) discovered EDTA-induced marginal band shrinking was short lived but could be maintained if fresh EDTA was added to PRP every hour [384].

In contrast, from the same study, platelet marginal band diameter increased when incubating human PRP (originating from citrate blood) for 6h. From these findings, it was predicted the platelet marginal band is of a constant number of microtubule

bundles thus, larger platelets have a thinner microtubule coil with a greater diameter than smaller platelets. During preplatelet/barbell transformation, preplatelets elongate through microtubule elongation. As preplatelets elongate they become barbell-shaped through acto-myosin-spectrin compression. Therefore, large yet thin marginal bands have the capacity to overcome microtubule elastic bending forces and acto-myosin-spectrin compression forces.

The above findings may explain why barbell platelets are only present in 37°C citrate blood. However, in our current study the total platelet area (determined by FITC CD61 fluorescence morphology) in 37°C citrate blood was no different than that observed at room temperature (data not shown). Unlike Thon's 6h PRP observations, our quantitation was conducted after incubating citrate blood for 15 minutes. Furthermore, size measurements were generated from CD61 fluorescence and not tubulin-labelled marginal bands.

Another possible explanation for the absence of barbell platelet in EDTA is defective $\alpha_{IIb}\beta_3$ signalling. Platelets incubated in EDTA for 30 minutes at 37°C display a dysfunction in $\alpha_{IIb}\beta_3$ signalling and lose their ability to associate with fibrinogen and as a result fail to aggregate in response to ADP stimulation [435-437]. This adverse effect is irreversible and through crossed immunoelectrophoresis (CIE) [438], sucrose density gradient sedimentation [439] and mab binding assays [440] has been shown to be a result of $\alpha_{IIb}\beta_3$ dissociation. Further examination has revealed this phenomenon does not occur in GT platelets associated with absence of $\alpha_{IIb}\beta_3$ and when platelets are preincubated with antibodies which target the α_{IIb} subunit [435].

Interestingly, when exposing 37°C citrate blood to eptifibatide, we show that barbell platelets were absent (Figure 4.15). As eptifibatide binds to the RDG binding motif of

the β_3 unit and CD61 also binds to β_3 , it was important to confirm the absence of barbells was not due to loss of CD61 fluorescence along the barbell shaft. However, as CD61 MFI of platelet with or without eptifibatide treatment did not decrease (Figure 4.15e) and no barbells were detected using BF imaging (data not shown), this suggested $\alpha_{IIb}\beta_3$ signalling may be involved in proplatelet/barbell conversion. Normal $\alpha_{IIb}\beta_3$ outside-in signalling has been shown to be essential for initiating MK proplatelet formation [186]. Furthermore, dysfunctional MK $\alpha_{IIb}\beta_3$ outside-in signalling results in deformities in proplatelet morphology and therefore macrothrombocytopaenia [267, 273, 274, 441-443]. As our data provides no information regarding the effects of eptifibatide on proplatelet/barbell platelet kinetics it is difficult to confirm whether eptifibatide inhibits barbell platelet formation completely or delays onset. To address this, barbell kinetic studies will be further explored in Chapter 5 when determining proplatelet/barbell platelet kinetics.

Like EDTA, barbell platelets were also absent in hirudin blood irrespective of temperature (Figure 4.1 & 4.8). Unlike EDTA and citrate, hirudin is a salivary derivative from the medicinal leech *Hirudo medicinalis* which inhibits thrombin through direct association thus, preventing clotting. Normally, hirudin anticoagulation is used to maintain normal calcium levels to perform platelet function testing. Hirudin has been shown to have no effect on proplatelet formation [444]. Heparin forms a complex with antithrombin which ultimately binds to and inhibits thrombin [445]. Within the same study, heparin was also shown to facilitate normal proplatelet formation.

Lack of barbell platelets in hirudin blood is in agreement with the observations from Thon *et al* (2012) where these were also absent in heparinised blood along with platelet marginal band contraction [384]. Although it is unlikely thrombin signalling is involved

in preplatelet/barbell conversion, thrombin has been shown to be optimal for causing phosphorylation of dynein [446].

4.4.3 Tubulin Structure During Preplatelet/Barbell Conversion

Super high-resolution SIM microscopy of α -tubulin labelled PRP demonstrated barbell platelets (originating from 37°C citrate whole blood) exhibit a continuous figure-of-eight tubulin cytoskeleton interconnected by a cytoplasmic bridge (Figure 4.11 & 4.12). Microtubules extend up through the cytoplasmic shaft and loop back at opposing ends to form two teardrop-like distal ends. In some cases, these elaborate structures displayed tandem arrays along the shaft. These features of preplatelet maturation have been observed by others [380, 382] and are analogous to MK proplatelets [163].

In contrast, large circular preplatelets contain a normal resting platelet marginal band morphology ($>3\mu\text{m}$ in diameter). In addition to barbell platelets, under these conditions, artefactual barbell-shaped microaggregates were also present. These structures were shown to consist of two adjacent platelet structures containing individual marginal bands.

Low resolution microscopy of α -tubulin labelled PRP (originating from 37°C citrate blood) showed insights into preplatelet/barbell morphogenesis. In addition to preplatelets and barbell platelets were elongated oval structures which seem to represent an intermediate stage of conversion (Figure 4.11). From these images, some oval structures were contracted at the midzone. This was confirmed in detail by SIM (Figure 4.12).

The observations of our study match that observed by Thon and colleagues (2012) when identifying preplatelet maturation in human blood for the first time [384]. From

this study, a mathematical model of barbell conversion was developed. These researchers' imaged barbell conversion in real time showing as preplatelets elongate, the peripheral microtubule bundle extends whilst the area of the platelet remains unchanged through actomyosin-spectrin stability. Such increases in microtubule bundles cause the preplatelet to contract at the midsection until adjacent microtubule bundles contact, where it is hypothesised microtubule associated proteins crosslink forming a shaft connecting two distal platelets. At this point, the midsection of the shaft twists several times to form a barbell shape. In another study by the same group, preplatelets and their barbell derivatives underwent depolymerisation of tubulin at 4°C causing barbell platelets to revert into preplatelets which was reversed when re-heating to 37°C [380]. This suggests barbell conversion occurs because of microtubule polymerisation and sliding at the preplatelet midsection supporting the notion that motor proteins play an important role in barbell formation.

From our characterisation of preplatelet maturation, an ImageStream^X gating strategy was designed to discriminate and further characterise preplatelets and barbell platelets in whole blood (Figure 3.8). Using this method, the mean diameter of preplatelets (>3µm in diameter) was 3.4µm with a perimeter range between 13 and 24µm (Figure 4.13). Only one study has quantified the size distribution of preplatelets originating from a murine foetal liver MK culture system [380]. Using MetaMorph analysis, the size distribution of preplatelets included: 69% 2-4, 25% 4-6, 4% 6-8 and 2% 8-10µm in diameter agreeing with our findings in human that the majority of preplatelets are between 3 and 4µm. Although most mouse platelets are small (1-2µm in diameter) it appears both human and mice contain a subpopulation of large platelets (possibly immature) which can undergo fission.

Alternatively, the barbell platelet perimeter in our study ranged between 13 and 42 μm . Thon's (2012) mathematical modelling of preplatelet/barbell conversion predicts the force required to convert into and barbell structure is inversely proportional to the platelet area [384]. Thus, below a certain size threshold, the force generated becomes insufficient. This lower size threshold was determined as 3 μm in diameter. As the lower barbell platelet perimeter measured by ImageStream^X (13 μm) is comparable to that of preplatelets, we confirmed that barbell platelets originate from preplatelets >3 μm in diameter.

4.4.4 Quantifying Preplatelet Maturation Events in 37°C Citrate Whole Blood.

The overall percentage of barbells in normal human blood determined by ImageStream^X is low (~0.5%; Figure 4.14a), suggesting that preplatelet maturation is transient and likely to be enhanced under normal shear and turbulent forces within the vasculature [175, 176, 380, 384]. Despite this, the fraction of barbells observed in our study in human blood was still ten-fold greater than previous observations [384]. However, previous measurements were conducted in tubulin labelled PRP on slides using a laser scanning cytometry approach. This difference possibly reflects the disparities between ImageStream^X and microscopy in detecting rare, transient and fragile structures such as these [387, 389, 396]. Furthermore, our ImageStream^X method relies on simple, direct antibody labelling and no separation steps which require centrifugation. Investigations have shown platelet yield is ~20% higher in PRP originating from EDTA blood compared to citrate-based anticoagulants [447]. As a result, it is highly likely that preplatelets and barbell platelets were excluded through centrifugation and/or platelet clumping during the PRP sedimentation process.

In contrast, the fraction of preplatelets (circular platelets $>3\mu\text{m}$ in diameter) was $\sim 3.6\%$ matching that observed by Thon [384]. This suggests in our study, $\sim 4\%$ of blood platelets have the capacity to convert into barbells and undergo fission to divide into two smaller platelets. Furthermore, combined preplatelet and barbell fractions equated to the IPF measured in EDTA whereas in citrate blood, IPF was 0.5% fewer suggesting barbells are excluded from the IPF measurement (Figure 4.14e). This suggests the IPF measurement in EDTA normally includes preplatelets and sphered barbells. The IPF measurements in our study matched that observed by others [362, 363]. The individual and combined fractions of preplatelets and barbell platelets positively correlated with IPF in normal blood (Figure 4.14b, c & d). IPF is thought to represent the rate of platelet production in health and disease with the potential to diagnose thrombocytopenia with varying aetiology [422, 448-450]. Size alone is limited in its capacity to determine thrombopoiesis across multiple thrombocytopenic disorders [342]. As a result, quantitation of preplatelets would share similar limitations. However, if preplatelets are indeed immature platelets, it is possible quantitation of barbells could be a more useful representation of the rate of thrombopoiesis. The analogy of preplatelets/barbells and immature platelets and their role in thrombocytopenia is explored in detail in Chapters 5 and 6.

The concentration of preplatelets, barbell platelets and IPF was measured up to 3h post phlebotomy at 37°C to determine any short-term changes in their respective kinetics. Although each population appeared to reduce slightly over time this was not significant (Figure 4.15a). It is likely that longer incubation times are required to determine kinetics as others have attempted to measure preplatelet division in washed platelets up to 6h incubation [382]. Indeed, this study yielded much higher

concentrations of barbell platelets compared to our findings in whole blood and Schwartz and co-workers (2010) were the first to describe platelets forming progeny through barbell morphogenesis [382]. From this study, platelets stored for transfusion or washed platelets from healthy donors were incubated at 37°C for 6h in serum-free M199 media under gentle agitation. Microscopy was used to quantify barbell platelets at 6h and concluded that approximately 5% of platelets underwent barbell transformation. However, the concept of large preplatelets forming barbells had yet to be described.

More recently, preplatelet division has also been measured in platelets captured within a semisolid viscous medium [451]. As platelets incubated in this medium remain static one can assume when preplatelet division takes place the formed progeny remain in close contact to one another. Furthermore, by labelling two separate washed platelet suspensions with two different cytosolic proliferation tracking dyes prior to mixing them together this group used confocal microscopy to quantify the number of single colour platelet singlets and doublets and those double positive for both tracking dyes. From this, a formula was generated where the relative number of two-coloured doublets was compared with the binominal prediction to quantify platelet division. From this analysis, it was predicted 8.8 -17.5% of platelet were capable of division which is much higher than that observed in our study.

Although the number of barbell platelets in washed platelet incubation assays appear exacerbated, researchers have never used this method to establish the kinetics of preplatelet maturation. Taking this into consideration, in chapter 5, this method will be utilised in conjunction with ImageStream^x to determine changes in preplatelet and barbell platelet quantification over time. Furthermore, in chapter 5, the use of cytosolic

cell proliferation dyes will enable individual dividing platelets to be tracked and elucidate whether progeny are formed.

4.5 Limitations and Future Directions

An important limitation regarding the optimal and accurate measurement of barbell platelets was potential contamination with barbell-shaped microaggregates. So, a key part of this study was to characterise these, determine their origin and develop a method to eliminate them from the analysis. CD62p labelling demonstrated that one or both aggregates were activated and could be simply excluded through CD62p positivity. Although this proved effective at removing these from analysis, some (albeit very few) coincidence events were still present within the barbell platelet population but were not significant and did not interfere with measurements. Thus, sample quality is essential for accurate barbell analysis.

Although our analyses are performed directly in diluted whole blood and careful steps have been taken to remove barbell-shaped microaggregates from the barbell platelet analysis, anticoagulation was required. Unfortunately, as the major function of platelets is to detect sites of vascular injury, become activated and rapidly change shape to support haemostasis and thrombus formation, some form of anticoagulation is necessary. The function of the anticoagulants used in this study have been mentioned in detail and each has obvious advantages and disadvantages in platelet research. Though it is possible that preplatelet maturation events measured in blood could be an artefact of trisodium citrate anticoagulation, the results are not only in agreement with but have extended the observations by Schwertz *et al* and Thon *et al* [380, 382, 384] in citrated samples. Disappointingly, this study has shown that hirudin anticoagulation

is not suitable for measuring preplatelets and barbells in agreement with the heparin observations by Thon *et al* [384]. This suggests not only that thrombin generation is not important for preplatelet to barbell conversion but that other agonists are causing microaggregate formation both in hirudin and perhaps in citrated anticoagulated blood. Further studies are required to potentially inhibit other agonists e.g., ADP using apyrase or P2Y12 inhibition to prevent aggregate formation.

As barbell platelets consist of an elaborate and delicate figure-of-eight morphology it is possible, processes such as hydrodynamic focussing of cellular events during ImageStream^x acquisition or even pipetting could generate enough shear force to break apart barbell platelets and therefore affect their quantitation. Also, considering the shape of barbell platelets, orientation of barbell platelets may also affect their quantification and characterisation by microscopy.

Orientation is also an important limitation for preplatelet quantification as it disregards platelets imaged as flattened discs, although it is thought that platelets undergo sphering during flow cytometry. Preplatelets also lack complete characterisation and can only be quantified based upon their size and diameter. Immature platelets are also normally thought to be larger than mature platelets [452]. However, using platelet size as a discriminator of platelet age has its limitations [342]. Lack of standardisation between instruments and EDTA-induced time dependent platelet swelling mean that this method has little clinical use. Although MPV is useful for diagnosing macrothrombocytopaenia it cannot measure subpopulations and fails to discriminate patients with either normal or BM failure [453, 454]. Although it has been shown MPV has some utility in measuring newly formed platelets, under resting conditions not all young platelets are large [343].

This chapter has presented an overview of marginal band morphogenesis during preplatelet/barbell conversion. However, microscopy-based characterisations were all conducted using static imagery. Realtime imaging of preplatelet barbell conversion would assist in further elucidating and characterising this process. In addition, although the morphological intermediate phases of preplatelet maturation (elongated preplatelets and barbell platelets) were identified, this approach lacked information regarding kinetics of preplatelets and barbells over extended periods of time. Furthermore, the results from our kinetic study in whole blood were inconclusive. Investigating the kinetics of preplatelet maturation remains a key aspect of this thesis and is therefore explored further in Chapter 5. Preplatelets were also identified using this method based on size. As immunofluorescence was performed using PRP centrifuged onto poly-L-lysine coated coverslips it is likely platelets become spread and may have appeared larger.

Finally, quantification of preplatelets and barbell platelets in normal human blood was conducted with 12 healthy individuals and compared to IPF measurements performed by the Sysmex XN1000 analyser. In order to generate truly meaningful data regarding preplatelets and barbell platelets in normal human blood a much larger cohort is required. However, from this data there is a clear relationship between preplatelets/barbell platelets and the IPF% suggesting quantification of preplatelet maturation events (barbell platelets) in whole blood may hold some potential to measure immature platelets to determine platelet production in health and disease. Further investigation in Chapter 5 also determines the role preplatelets and barbell platelets play in haematological disorders related to thrombocytopenia. Furthermore, if preplatelets and barbells are indeed analogous to RP then it is highly likely they

represent immature platelets which undergo further terminal maturation via fission which will be explored in Chapter 5 & 6.

4.6 Conclusions

The preservation of preplatelet maturation events in whole blood was dependent on temperature (i.e., 37°C) and anticoagulation (i.e., trisodium citrate). The fraction of preplatelets and their barbell derivatives equate to current immature RP measurements (determined by IPF) in EDTA blood. As the fraction of barbell platelets correlated with the IPF% in normal EDTA blood, subsequent chapters will further determine whether barbells not only originate from newly formed platelets but can undergo terminal maturation and fission. Their physiological role(s) in normal and thrombocytopenic conditions will also be further explored.

Chapter 5 - The Thrombopoietic Significance of Preplatelet Maturation

5.1 Introduction

In Chapter 4, it was established that preservation of preplatelet maturation in human whole blood is dependent on 37°C temperature and citrate anticoagulation (Figure 4.1 and 4.8 - 4.10). Furthermore, using these conditions, advanced microscopy in combination with ImageStream^X was utilised to characterise and quantify preplatelet maturation events in normal blood samples (Figure 4.11 - 4.13 and 4.14a). From this, it was concluded, in healthy control blood, preplatelets and barbell derivatives appear to display similar thrombopoietic indicators as immature RP (Figure 4.14b) and therefore, measurement of these maturation events potentially represents the rate of platelet production across thrombocytopenic conditions.

RP and IPF measurements have largely failed to become established within clinical settings due to issues with standardisation (such as variables regarding specificity of nucleic acid dyes) [353, 355]. Furthermore, when investigating the significance of RP, it appears this method is limited by disease context and often fails to discriminate between normal and patients with BM failure [421]. As a result, apart from platelet counting there are no non-invasive methods which currently aide diagnostics and management of thrombocytopenia. More recently, attempts have been made to improve nucleic acid dye stability using SYTO 13 [371]. Also, alternative advances such as HLA I expression and the number of mitochondria has been suggested as markers of platelet age [373, 455]. However, neither of these markers are platelet specific unless used in conjunction with specific antibodies and flow cytometry. Furthermore, they fail to completely resolve the immature platelet population from the mature. Increases in MPV also occur concomitantly with episodes of thrombocytopenia [452]. We therefore hypothesise that preplatelets are the true newly formed immature

RP which are increased in response to consumption or enhanced peripheral destruction (an aetiology associated with ITP). Although others have suggested that preplatelets can undergo terminal maturation by fission within the circulation [380], no investigators have yet to study preplatelet maturation in thrombocytopenia. High throughput morphometric quantification of preplatelet maturation may offer a promising new approach to accurately determine platelet immaturity and the rate of platelet production across thrombocytopenic conditions.

If preplatelets are analogous to immature RP, then what is their physiological role in the blood? One study has shown, preplatelets and MK proplatelets isolated from a murine foetal liver MK culture system increase the platelet count of mice 300% more than normal platelet transfusions [380]. Also, others have shown incubated washed platelets stored at 37°C under standard blood bank conditions increase platelet counts on average by 21.1% (+/-5.1%) after 5 days [382]. Thus, it is highly likely, preplatelet maturation takes place in the bloodstream to increase platelet counts. However, it is unclear if preplatelets have the capacity to convert into barbells either once or multiple times until reaching the lower diameter threshold of 3µm hypothesised by Thon and colleagues (2012) [384] and also confirmed in our study (Figure 4.13b).

Cytoskeletal reorganisation has been shown to be essential during preplatelet/barbell conversion [9, 382]. Despite this, little is known regarding how this phenomenon is regulated and if preplatelets need to generate the energy requirements to undergo such elaborate transformations. In Chapter 4, it was highlighted $\alpha_{IIb}\beta_3$ as a potential regulator of cytoskeletal reorganisation required during barbell conversion as no barbells were present in citrated blood containing eptifibatide (Figure 4.15d).

Furthermore, $\alpha_{IIb}\beta_3$ outside-in signalling has been shown to be essential for initiating MK proplatelet formation [186] through its association with FLNA [111].

We hypothesise that preplatelets are analogous to newly formed immature platelets which can mature into smaller platelets in the circulation to increase the platelet count. Therefore, high throughput morphometric quantitation of these maturation events offers a new approach to accurately measure immaturity to indirectly determine the rate of platelet production across thrombocytopenic conditions.

Therefore, the objectives of this chapter were:

1. To determine whether preplatelets and barbell platelets measured by ImageStream^X are analogous to newly formed immature RP.
2. To determine the physiological importance of preplatelet maturation and how it is regulated.
3. To establish whether platelet maturation events represent the rate platelet production in acquired thrombocytopenia associated with enhanced peripheral destruction or in the absence of BM production.

5.2 Methods

For all fluorescence analyses, except SiR tubulin, samples were fixed with 2% formalin prior to ImageStream^X acquisition. To determine if preplatelets and barbell platelets express markers of platelet ageing, healthy control citrated blood (n=7) was incubated for 30 minutes at 37°C in PBS containing anti-human BV421 CD42b, FITC TO

(200ng/mL), PE CD62p (1/100), APC HLA I (1/100) and AF599 Mitotracker CMXRos (5 μ M) (see Table 2.3 for antibody, dye & probe details). 10,000 BV421 CD42b platelets were then acquired using the ImageStream^X. Preplatelets and barbell platelets were discriminated by ImageStream^X (Figure 3.8) using BV421 CD42b fluorescence (instead of FITC CD61) and microaggregates were removed using PE CD62p (instead of BV421 CD62p). Localisation of platelet ageing markers was depicted using ImageStream^X galleries (x60 lens). Positive MFI signals were determined for TO and mitotracker using FMOs, for SSC by imaging platelets with the SSC laser turned off and for APC HLA I and PE CD62p using anti-mouse IgG2a (Clone: MOPC-173, BioLegend, #400220) and IgG1 (Clone: MOPC-21, BioLegend, #400112) κ isotype controls respectively. Compensation matrices were determined by acquiring 1000 images from each single stain control using 405, 488 and 647 lasers and BF and SSC switched off. Compensation matrices were generated in IDEAS.

From here in, unless otherwise stated, preplatelets and barbells were discriminated in citrate whole blood or washed platelets using the BV421 CD62p, FITC CD61 and PE CD42b antibody labelling and ImageStream^X IDEAS gating strategies described in chapter 3 (Figure 3.2 & 3.8). To establish whether preplatelet maturation events indirectly represent the rate of platelet production in acquired thrombocytopenia, patients with ITP (n=8) and those undergoing chemotherapy-induced BM ablation (n=6) were recruited and preplatelets and barbell platelets were determined in citrate whole blood to determined difference in conditions associated with enhanced and no platelet production respectively. Preplatelet and barbell diameter and perimeter measurements were determined using an erode+4 of FITC CD61 fluorescence. For ITP samples, PRP (n=5) originating from citrate blood was indirectly labelled with goat

anti-mouse α -tubulin, secondary labelled with rabbit anti-goat 488 (see section 2.12 in “General Methods”) and imaged using DM6000 Leica (x60 lens) to determine marginal band morphology of preplatelet maturation events.

To determine the physiological relevance of preplatelet maturation, an in vitro washed platelet incubation assay was designed. For differences in barbell formation in whole blood and washed platelet suspensions (n=5), comparable concentrations of platelets from citrated blood and washed platelets (originating from citrated whole blood) in serum-free M199 media were incubated for 3h at 37°C under constant gentle agitation (using a microplate mixer 300 rpm; StarLab N2400-8040) within a cell incubator (37°C; 5% CO₂, 12% O₂). The tubulin morphology and perimeter of washed platelet barbell platelets was determined by ImageStream^x using SiR tubulin labelling (0.4 μ M; incubated for 30 minutes) and a FITC CD61 erode+4 mask respectively (n=5). For preplatelet and barbell platelet kinetic studies, washed platelets (100 x 10⁶/mL) in M199 media were incubated for 24h under the same conditions above (n=7). For the dual cytosolic labelling experiment, two separate washed platelets (100 x 10⁶/mL) suspensions were labelled with either CellTraceTM CFSE (0.2 μ M; emission 517nm; ThermoFisher, #C34570) or far red (1 μ M; emission 661nm; ThermoFisher, #C34572) cytosolic dyes for 30 minutes, centrifuged at 1000g for 10 minutes with 1 μ M PGI₂ to remove precipitates (within the supernatant). Labelled washed platelets (100 x 10⁶/mL) were pelleted in M199 media, mixed and incubated as above (n=3). Alternatively, to track preplatelet division events, washed platelets were all labelled with CellTraceTM yellow (2 μ M; emission 579nm; ThermoFisher, #C34573). As CellTraceTM yellow is detected in the PE channel, 10,000 platelet images were acquired from FITC CD61 fluorescence only instead of the usual FITC CD61/PE CD42b positive approach

described in Figure 3.2. TRAP-induced degranulation was inflicted on CellTrace™ yellow-labelled washed platelets to determine if reductions in cytosolic dye intensity is influenced by granule release (see section 2.11 “General Methods”). When investigating the regulation of preplatelet maturation using CellTrace™ yellow, in addition to BV421 CD62p and FITC CD61 labelling, washed platelets were also labelled with mitotracker CMXRos (0.5mM) as part of the same 15-minute antibody incubation at 24h. Mitotracker MFI was determined by ImageStream^X using an FMO and compared between platelets and progeny formed following incubation. Inhibition of mitochondrial respiration (rotenone 3μM; n=5), platelet aggregation (eptifibatide 10μM; n=5) and cytoskeletal signalling (nocodazole 10μM, cytochalasin D 1 & 0.1μM; n=5) was performed using washed platelets incubated as above for 6h. To determine platelet activation following exposure to eptifibatide, washed platelets (n=5) were labelled with BV421 CD62p and MFI was determined by a BV421 anti-mouse IgG1 κ isotype control (Clone: MOPC-21, BioLegend, #400158). To observe platelet marginal band morphology following cytoskeletal disruption platelets were labelled with SiR tubulin as above (n=5).

5.3 Results

5.3.1 Preplatelets and Barbell Platelets are Analogous to Immature Reticulated Platelets

Immature RP have been shown to display an increase in nucleic acid content (determined by nucleic acid dyes) [349, 353], granularity [372], HLA I expression [373] and contain more mitochondria than old platelets [372, 455].

Preplatelet and barbell platelet nucleic acid content (TO), granularity (determined by SSC light) and HLA I and mitotracker expression were determined using ImageStream^x by MFI. Figure 5.1 shows, preplatelets and barbell platelets are positive for TO (a), HLA I (c) and mitotracker CMXRos (d) whilst displaying an intense SSC signal (b). TO images showed multiple positive regions within preplatelets and within the distal platelets of barbell platelets. No apparent TO signal was present along the barbell shaft. This was the same for mitotracker CMXRos localisation however, individual mitochondria were not visualised by ImageStream^x resolution (x60 lens). In contrast, HLA I was expressed throughout the PM of both preplatelets and barbell platelets (including the shaft).

To determine if preplatelets and barbell platelets consist of similar biomolecular composition as immature RP, the MFI of platelet ageing markers was quantified. Platelets were determined from the preplatelet histogram as circular BV421 CD42b positive platelet images <3µm in diameter (see Figure 3.8). Barbell platelets showed significant increases in all platelet ageing markers including: TO ($p = 0.0210$), SSC ($p = <0.0001$), HLA I: $p = 0.0145$ & mitotracker ($p = 0.0152$). In contrast, preplatelets also displayed significant increases in TO, SSC and mitotracker ($p = 0.0420$, 0.0027 & 0.0184 respectively) however, levels of HLA I were no different than normal platelets ($p = 0.1740$; Figure 5.2a-d).

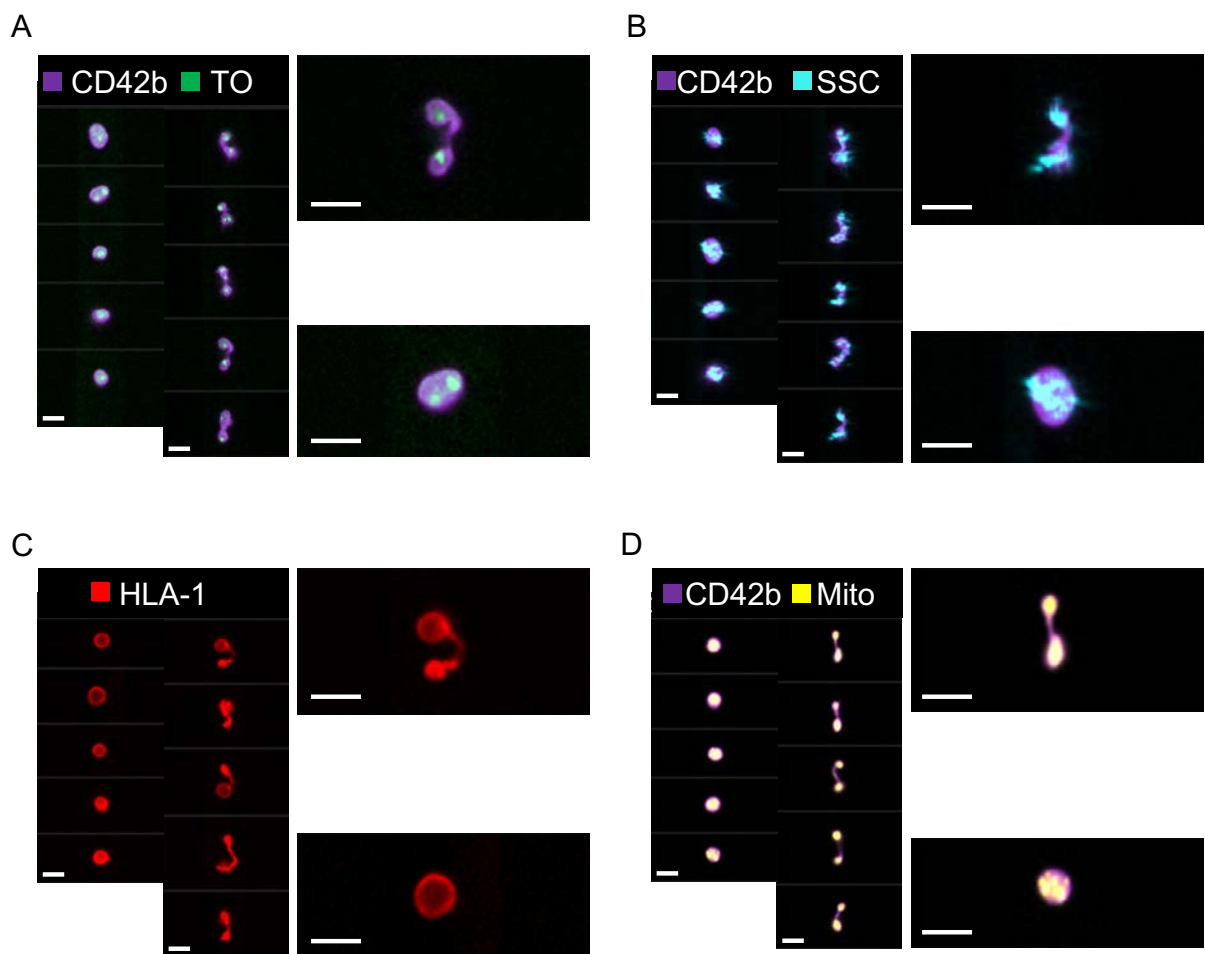


Figure 5.1: Localisation of Platelet Ageing Markers

Healthy control citrate whole blood was incubated for 30 minutes at 37°C with anti-human BV421 CD42b, AF512 thiazole orange (TO; 200ng/mL), PE CD62p, AF599 Mitotracker CMX Ros (5µM) and APC HLA I and 10,000 CD42b platelet images were acquired using the ImageStream^x (x60 lens). Preplatelets and barbells were gated using the method described in Figure 3.8 using BV421 CD42b and PE CD62p instead of FITC CD61 and BV421 CD62p. ImageStream^x galleries were created to show localisation of (A) nucleic acid content (TO; green), (B) granularity (cyan; determined by SSC light), (C) HLA I (red) and (D) Mitochondria (yellow) within CD42b positive (purple) preplatelets and barbell platelets (n=7). Figure is representative of one experiment. Scale Bar 7µm.

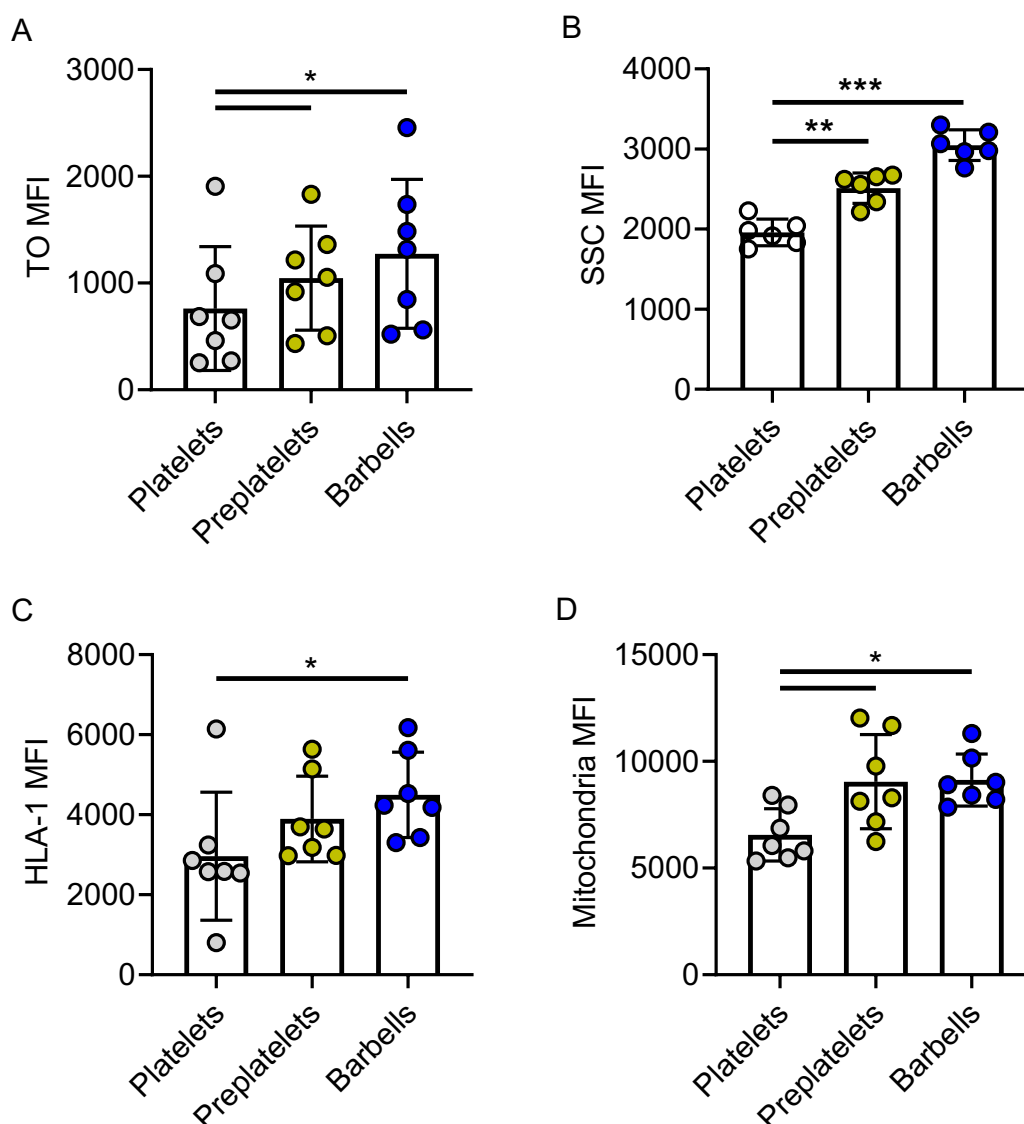


Figure 5.2: Preplatelets and Barbell Platelets Display a Greater Expression of Platelet Ageing Markers

Healthy control citrate whole blood was labelled as described in Figure 5.1 and 10,000 CD42b platelet images were acquired using the ImageStream^x to determine median fluorescence intensity (MFI) of (A) thiazole orange (TO; green), granularity (SSC; cyan), (C) HLA I (red), (D) mitochondria (yellow) within the CD42b (purple) positive platelet, preplatelet and barbell platelet populations (n=7). Data is normalised with platelet size. Error bars +/- 1 SD. (A-D) one way ANOVA with Dunnett's multiple comparison test. Sig. * <0.05 , ** <0.01 , *** <0.001 .

5.3.2 Barbell Platelets in Whole Blood Represent Immature Platelets and the Rate of Thrombopoiesis in Acquired Thrombocytopenia

Our data so far suggests preplatelet maturation is limited to immature RP ($>3\mu\text{m}$ in diameter) and the percentage of circulating barbells is therefore equivalent to immaturity and could therefore be used as an indirect measurement of the rate of platelet production. To confirm these findings, preplatelets and barbells were quantified by ImageStream^x (see Figure 3.8) in ITP citrated blood incubated at 37°C for 1.5h. ITP is a condition characterised by thrombocytopenia caused by enhanced peripheral immune destruction which induces significant increases in MPV and RP% (the latter determined by IPF%; Figure 5.3; $p = <0.0001$ for all comparisons).

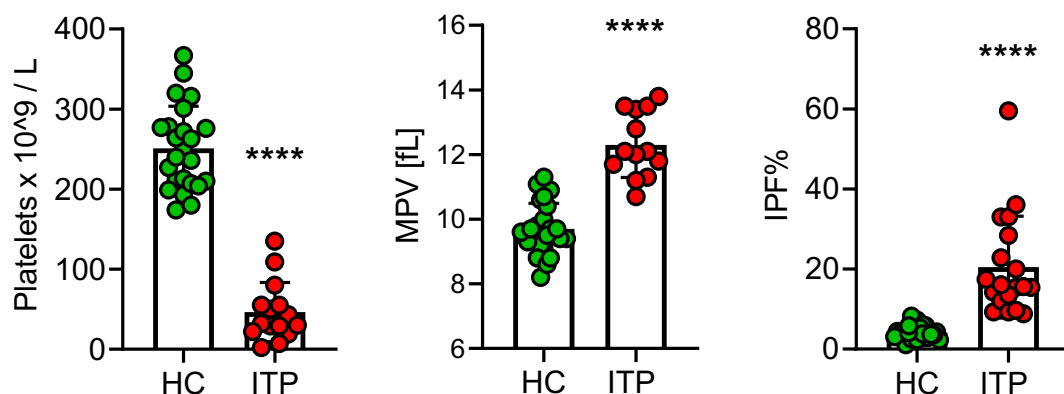


Figure 5.3: ITP Patient Platelet Count Data

Whole blood from healthy controls (HC) ($n=25$) and ITP patients ($n=15-19$) was anticoagulated with EDTA and full blood counts were measured using the XN1000 (Sysmex) haematology analyser to determine differences in platelet counts (determined by PLT-F), MPV and IPF%. Error bars ± 1 SD. Unpaired t-test. Sig. **** <0.0001 . PLT-F: platelet fluorescence, MPV: mean platelet volume; IPF: immature platelet fraction; ITP: immune thrombocytopenia.

Preplatelets and barbells were detectable by ImageStream^x in healthy control and ITP patient blood samples (Figure 5.4a & 5.4b respectively). additional images of healthy

control and ITP preplatelets and barbell platelets can be found in Appendix Figure 4.2 & 4.3 and 5.1 & 5.2 respectively.

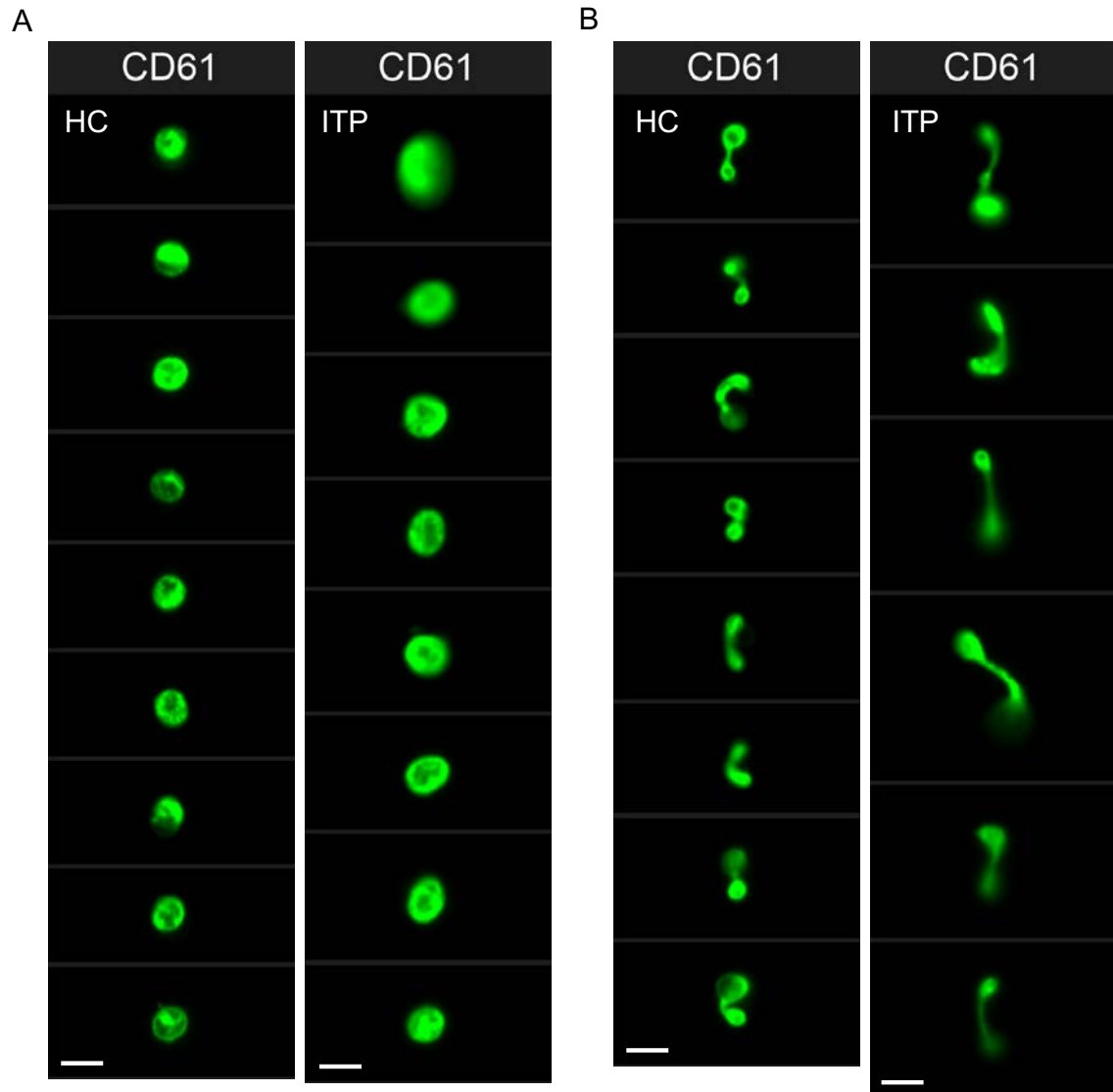


Figure 5.4: Preplatelets and Barbell Platelets in Healthy Control and ITP Citrate Blood
10,000 FITC CD61 positive platelet images were acquired by ImageStream^X (using the gating strategies described in Figure 3.2 and 3.8) from healthy control (HC) and ITP (immune thrombocytopenia) citrated whole blood. (A) Preplatelets and (B) barbells are depicted by FITC CD61 fluorescence. Scale bar 7 μ m.

The percentage of preplatelets and barbell platelets in ITP was significantly greater ($p = 0.003$ and 0.043 respectively) than controls (Figure 5.5a). Furthermore, both individual and combined percentages of preplatelets and barbells correlated with the IPF% (Figure 5.5c, 5.5d & 5.5e: Preplatelet/IPF: $p = 0.0005$; Barbell/IPF: $p = <0.0001$; combined preplatelet and barbell/IPF: $p = 0.0015$). However, in contrast to normal samples, the concentration of preplatelets and barbells did not differ (Figure 5.5b). The mean area (determined by FITC CD61 fluorescence morphology) of preplatelets and barbell platelets was also significantly larger in ITP than healthy controls ($p = 0.0026$; Figure 5.6).

Tubulin labelling of PRP confirmed that the frequency of barbell structures in ITP is much greater than in normal blood (Figure 5.7a; barbell platelets are indicated with blue arrows). Like in normal, PRP from ITP citrated blood contained barbell platelets consisting of the classical figure-of-eight barbell morphology and elongated preplatelet intermediates (Figure 5.7b). However, ITP barbells were more elaborate, forming larger elongated barbell platelets and MK proplatelet-like structures consisting of extended projections containing distinct cell bodies and tandem arrays. Intermediate elongated preplatelets were also much larger than those observed in healthy controls.

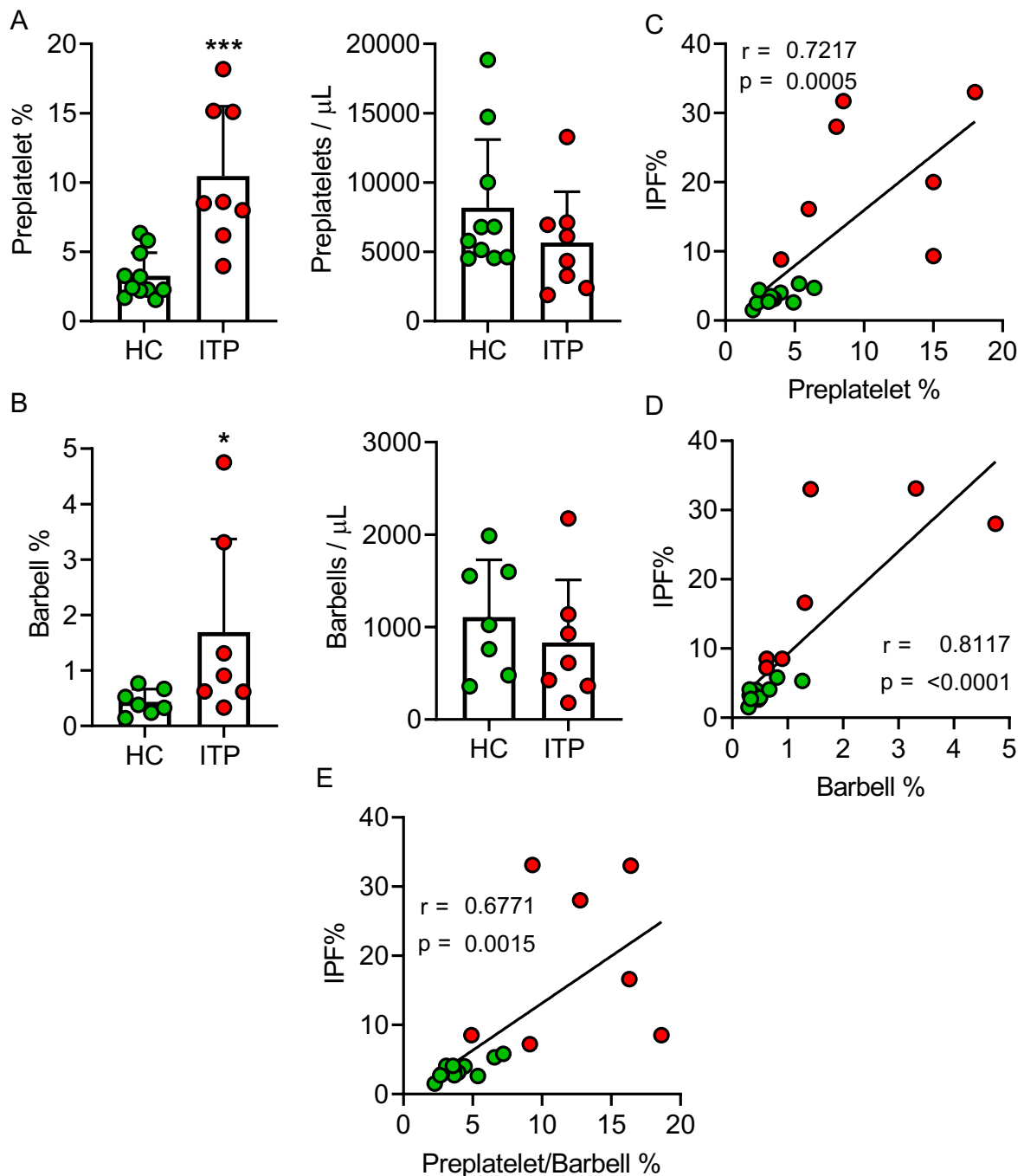


Figure 5.5: Preplatelet Maturation Events in ITP Citrated Whole Blood

All experiments were performed using ImageStream^X (x60 lens), acquiring 10,000 FITC CD61/PE CD42b positive platelet events (see Figure 3.2). Citrated healthy control (HC; n=8-12) and immune thrombocytopenia (ITP; n=7-8) whole blood was incubated for 1.5h at 37°C and (A) preplatelets and (B) barbell platelets were quantified from CD61 fluorescence (see Figure 3.8). Correlations between percentage (C) preplatelets, (D) barbell platelets or (E) combined preplatelets and barbells with IPF% (determined by XN1000, Sysmex) in healthy control and ITP citrate whole blood (n=7). Comparison between healthy control and ITP barbell platelet area determined by CD61 morphology (images depicted by brightfield and CD61 fluorescence; n=>8). Scale bar 7 μ m). Error bars +/- 1 SD. (A & B) paired t test, (C-E) Pearson's Correlation Coefficient, (D) unpaired t test. Sig. *<0.05, **<0.01, ***<0.001.

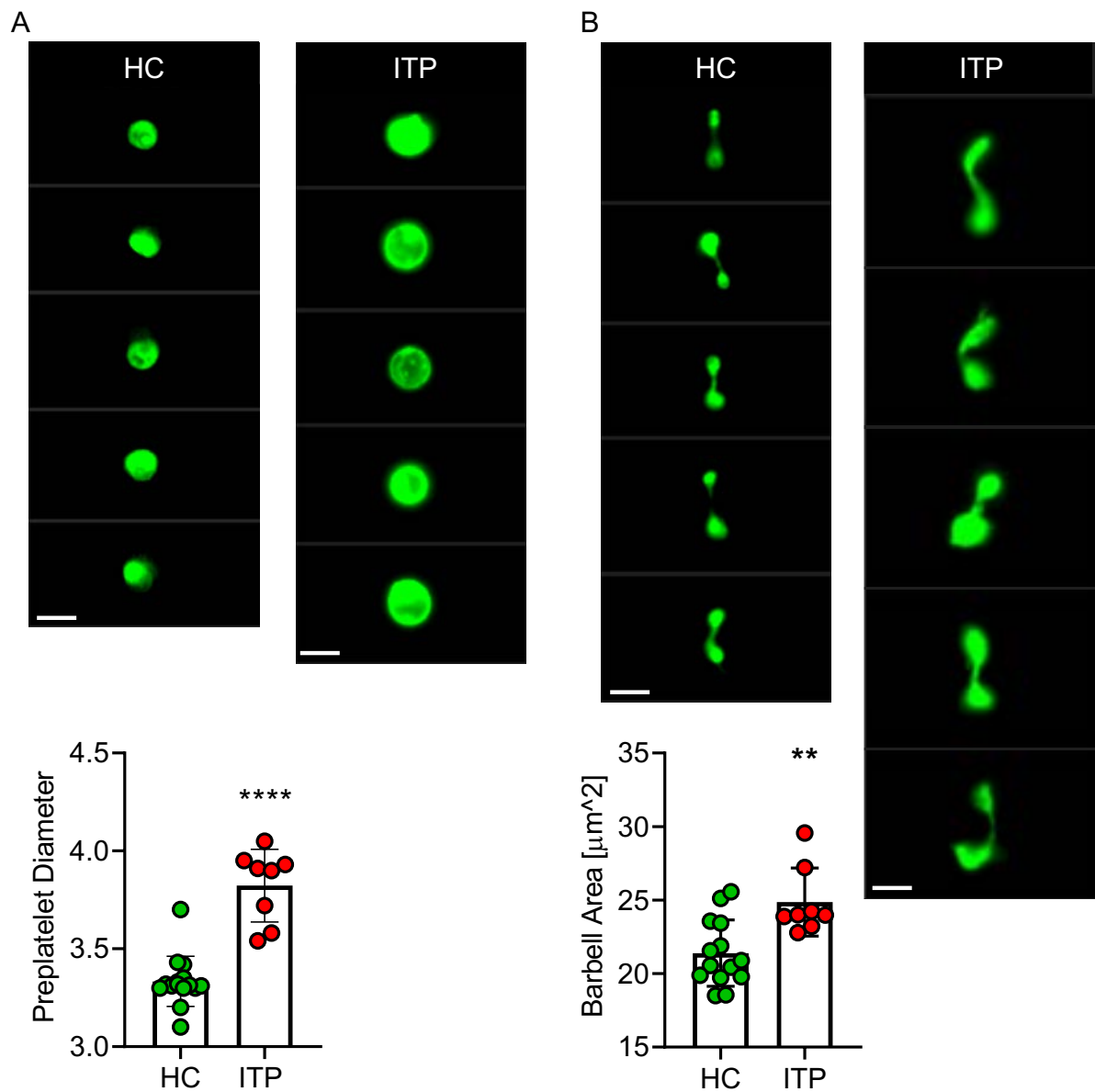


Figure 5.6: Preplatelets and Barbell Platelets are Significantly Larger in ITP Citrated Blood

All experiments were performed using ImageStream^x. Citrated healthy control (HC: n=15) and ITP whole blood (n=8) was incubated for 1.5h at 37°C. (A) Mean preplatelet diameter and (B) barbell platelet area in healthy control (HC) and immune thrombocytopenia (ITP) citrated blood measured using CD61 fluorescence morphology of an erode+4 pixel mask. ImageStream^x galleries are included to show differences between healthy control and ITP preplatelet and barbell platelets, depicted by CD61 fluorescence (green; scale bar 7μm). Error bars +/- 1 SD. Sig. **<0.01, ****<0.0001.

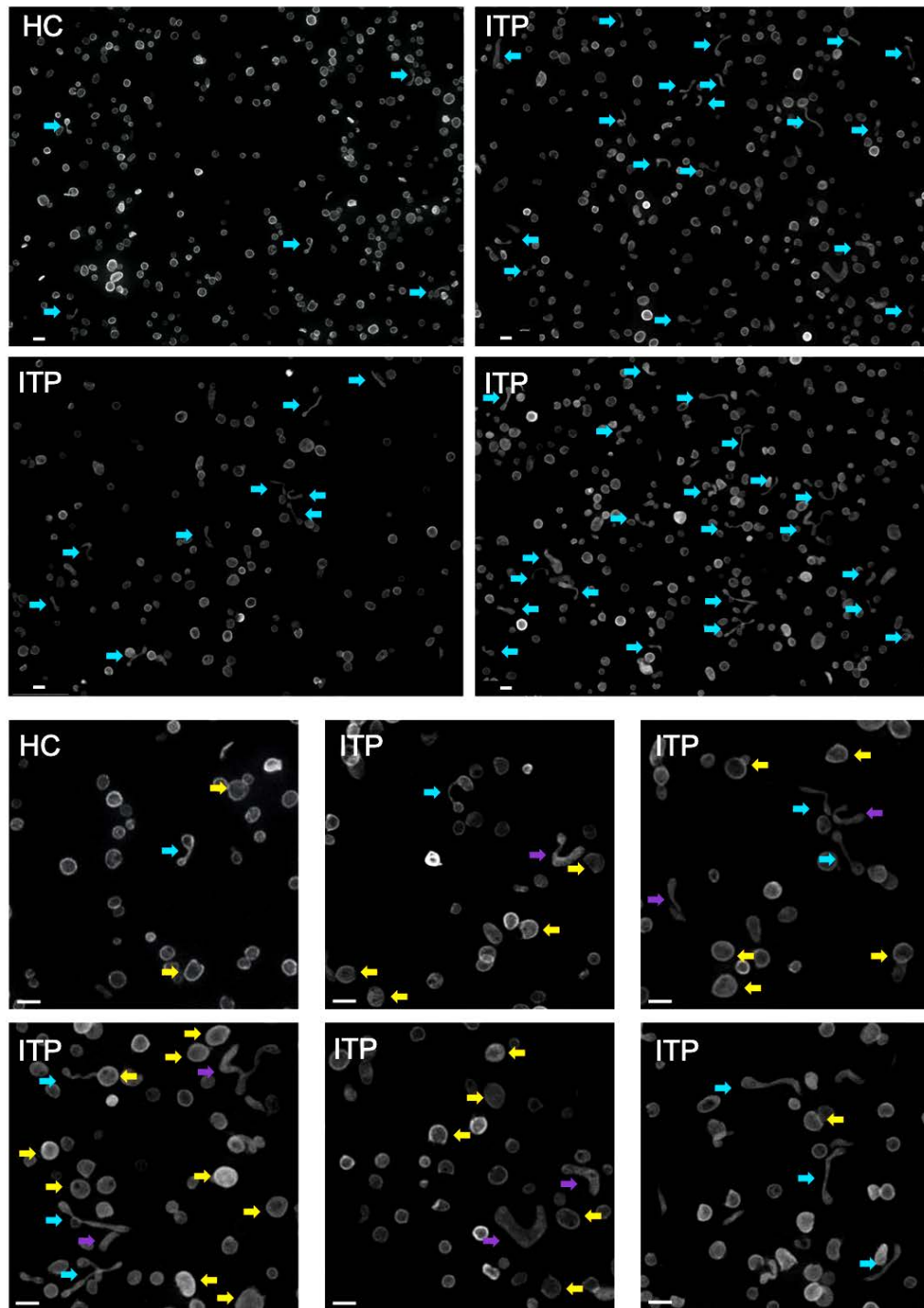


Figure 5.7: Determining Marginal Band Morphology of Preplatelet Maturation Events in ITP

(A & B) PRP ($5 \times 10^6/\text{mL}$) originating from healthy control (HC; $n = 1$) and immune thrombocytopenia (ITP; $n = 5$) citrate whole blood was incubated for 1.5h and fluorescently labelled for tubulin. Marginal band morphology was imaged using the Leica DM6000 widefield microscope (x60 lens; $n=5$). Examples of preplatelets are indicated by yellow arrows, elongated preplatelets by purple arrows & barbells by blue arrows. Scale bar $5\mu\text{m}$). Each image is representative of an individual donor.

Immature RP measurements are often limited in their ability to discriminate between conditions of normal and suppressed BM activity [421]. To determine the sensitivity of quantifying preplatelet and barbell platelets in citrated blood, samples were taken from patients (n=6) with lymphoma or myeloma 6 days post BM ablation (nadir) with chemotherapy. The fraction of preplatelets and barbell platelets were compared to baseline (prior to chemotherapy) measurements using ImageStream^x (described in Figure 3.8). See Figure 5.8 for chemotherapy treatment time course and platelet counts.

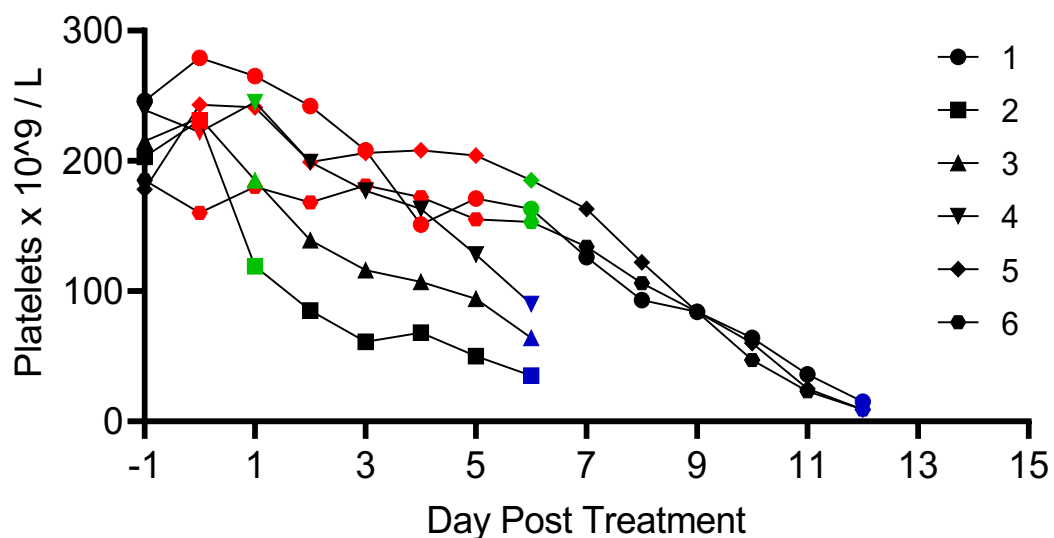


Figure 5.8: Platelet Counts Following Chemotherapy-Induced Bone Marrow Ablation

Patients with myeloma or lymphoma (n=6) were administered either a single dose of melphalan or 6 doses of LEAM (signified by red) and by given a blood stem cell transplant (green) the day after the final treatment. Blood samples were taken 6 days post-transplant (blue) where blood counts (XN1000 Sysmex) and preplatelets and barbell platelets were measured (ImageStream^x; Figure 3.8). Blood samples were also taken prior to chemotherapy (day -1) to determine baseline measurements. Each data line represents an individual patient.

Baseline platelet counts and IPF% were normal at $228 \times 10^9/\mu\text{L}$ and 3.5% respectively (Figure 5.9a & 5.9b). Preplatelet and barbell platelet levels were also normal at 4.2 and

0.4% respectively (Figure 5.9c & 5.9d). Post chemotherapy, as expected, platelet counts (33 x 10⁹/μL), were significantly lower than baseline (p = <0.0001). Although, despite both IPF% and the percentage preplatelets remaining normal (2 %: p = 0.0720 and 3.7%: p = 0.1663 respectively), barbell formation was highly significantly reduced (0.07%; p = <0.0001; Figure 5.9d).

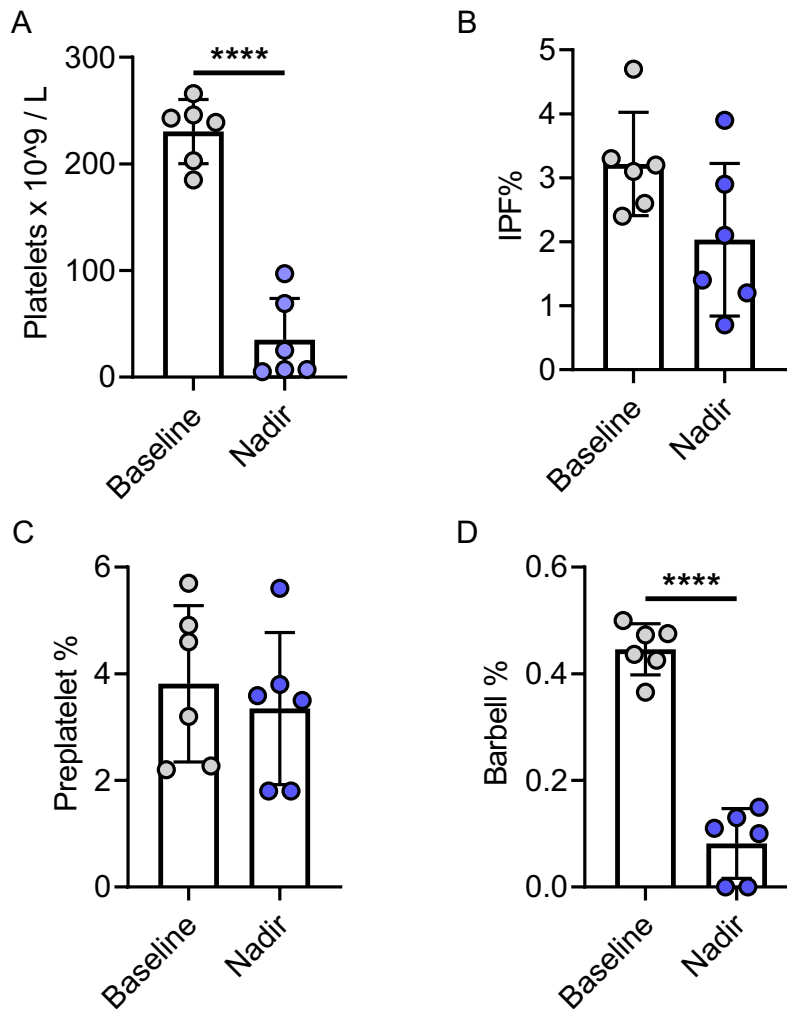


Figure 5.9: Quantifying Preplatelet Maturation Events Following Bone Marrow Ablation
Patients (n=6) receiving chemotherapy and blood stem cell transplant were recruited prior to therapy (baseline) and 6 days post-transplant (nadir). (A) platelet counts (PLT-F) and (B) IPF were determined by XN1000 (Sysmex). (C) Preplatelet and (D) barbell platelet percentage were measured by ImageStream^x (see Figure 3.2 and 3.8). (A-D) unpaired t test. Sig. ****<0.0001.

5.3.3 Preplatelets Mature into Barbell Platelets and Undergo Multiple Rounds of Fission to Increase the Platelet Count

To determine the physiological relevance of preplatelet maturation, *in vitro* kinetic studies were replicated from the paper by Schwartz and colleagues 2010 [382]. These researchers found ~5% of washed platelets transformed into barbell structures when incubated up to 6h at 37°C in serum-free M199 media whilst being maintained under gentle agitation. This is much higher than the ~0.5% of barbell observed in chapter 4 when incubating whole blood for 3h (Figure 4.15a). Thus, preplatelet barbell transformation appears to be enhanced under Schwartz's conditions.

To confirm this, human control citrated blood and equivalent concentrations of washed platelets (from a single donor; n=5) in M199 media were incubated at 37°C for 3h and preplatelets and barbell platelets were quantified at 0, 1.5 and 3h by ImageStream^X (Figure 3.8). The percentage of barbell platelets in citrated blood remained stable at 37°C 0-3h hours post phlebotomy (Figure 5.10a). In contrast, washed platelet barbells (see Appendix Figure 5.3 for ImageStream^X Galleries) increased in a time dependent manner and at 3h was ~4X greater than in whole blood relative to the total platelet count (Figure 5.10a). Using the same ImageStream^X IDEAS method described in Chapter 3 (Figure 3.8), the perimeter of washed platelet barbells was determined from a CD61 erode+4 mask. These barbells consisted of a perimeter range between 13 and 41 suggesting they originated from preplatelets >3µm in diameter (Figure 5.10b) and contained a figure-of-eight-shaped marginal band (depicted by SiR-tubulin labelling; Figure 5.10c & Appendix Figure 5.4). Furthermore, dual labelling of washed platelets with either cytosolic CellTraceTM CFSE (green) or far-red cytosolic dyes also clearly

demonstrated that barbell platelets indeed originate from single platelets confirming they are not an artefact of aggregation (Figure 5.10d).

As whole blood cannot be incubated for extended periods due to red cell lysis along with platelet activation and leukocyte aggregate formation, the kinetics of preplatelet maturation events was extended and investigated within washed platelet preparations described above. To further characterise preplatelet transition into barbell formation and eventual fission in vitro, the incubation was extended to 24h and the ImageStream^X “barbells” gate (Figure 3.8) was interrogated at 0, 1.5, 3, 6 and 24h. Figure 5.11a demonstrates barbell formation occurs at 1.5, 3 and 6h but is completely absent at baseline and 24h. Furthermore, preplatelet number also significantly decreased ($t=6h$: $p = 0.0002$) with the observed increase in formed barbell structures ($t=3h$: $p = 0.0175$; Figure 5.11b) suggesting the incubated preplatelet population is undergoing barbell formation and subsequent fission by 24h. Furthermore, IPF also significantly decreased to a similar extent to preplatelets (data not shown). However, it cannot be ruled out that nucleic acid content degraded during the incubation process and therefore artefactually reducing the IPF.

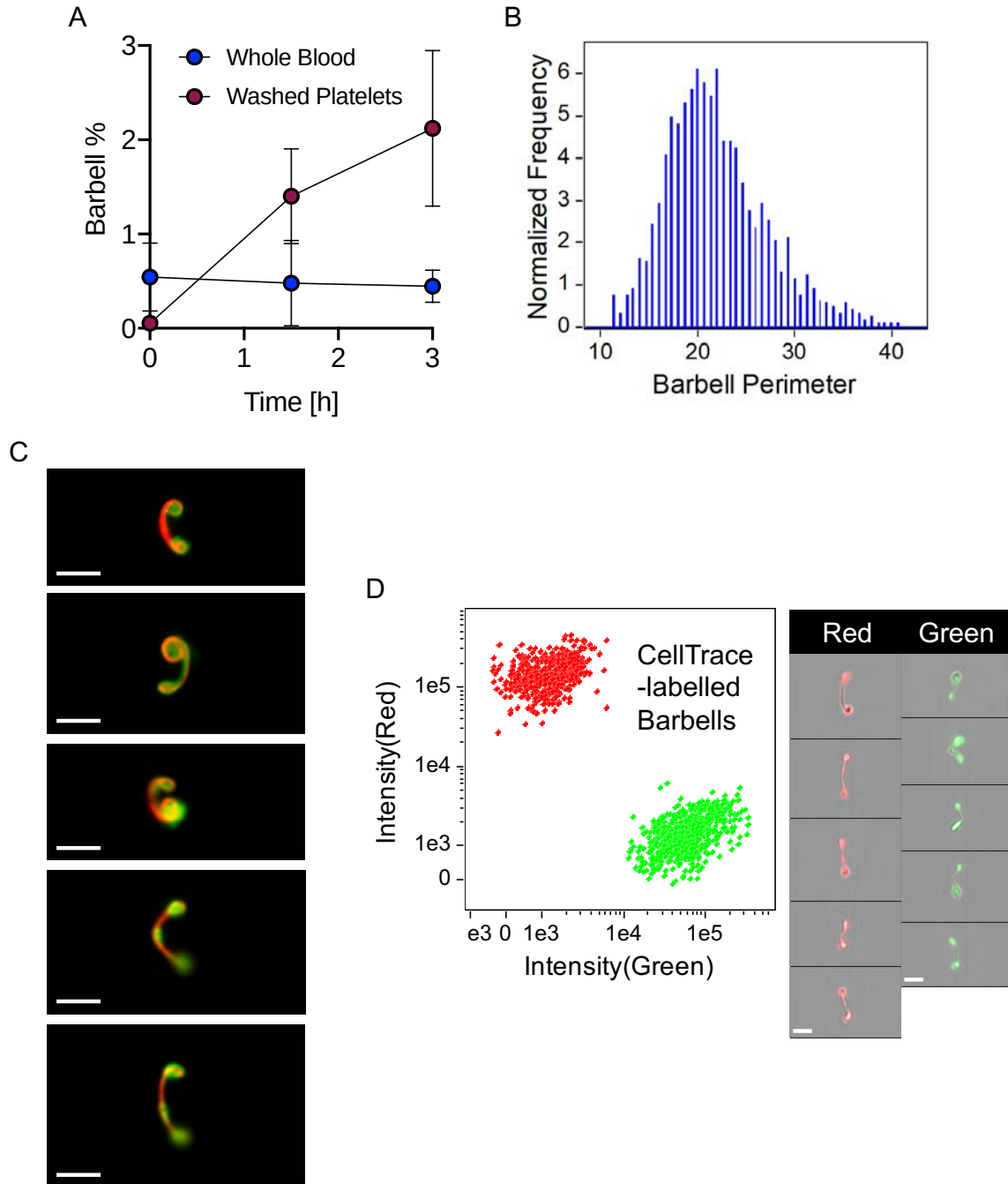


Figure 5.10: Preplatelet Barbell Conversion is Enhanced when Incubating Washed Platelets from Citrate Blood

Barbell platelets were discriminated as in Figure 3.8. (A) Citrate blood and equal concentrations of washed platelets from a healthy donor (n=5) were incubated for 3 hours at 37°C under gentle agitation and the percentage barbell platelets were quantified by ImageStream^X at 0, 1.5 & 3h. (B) Perimeter range of washed platelet barbell platelets determined by ImageStream^X using a CD61 erode+4 mask (n=5). (C) Washed platelets incubated for 3h at 37°C were labelled with SiR tubulin (0.4μM) and imaged using ImageStream^X (n=5). (D) 100 x 10⁶/mL washed platelets were labelled with either CellTraceTM CFSE (green) or Far-Red cytosolic dyes, mixed together and incubated for 3h (n=3). Barbell platelets were discriminated using ImageStream^X and cytosolic labelling was interrogated within the barbell gate (n=3). Scale bar 7μm. Error Bars +/- 1 SD.

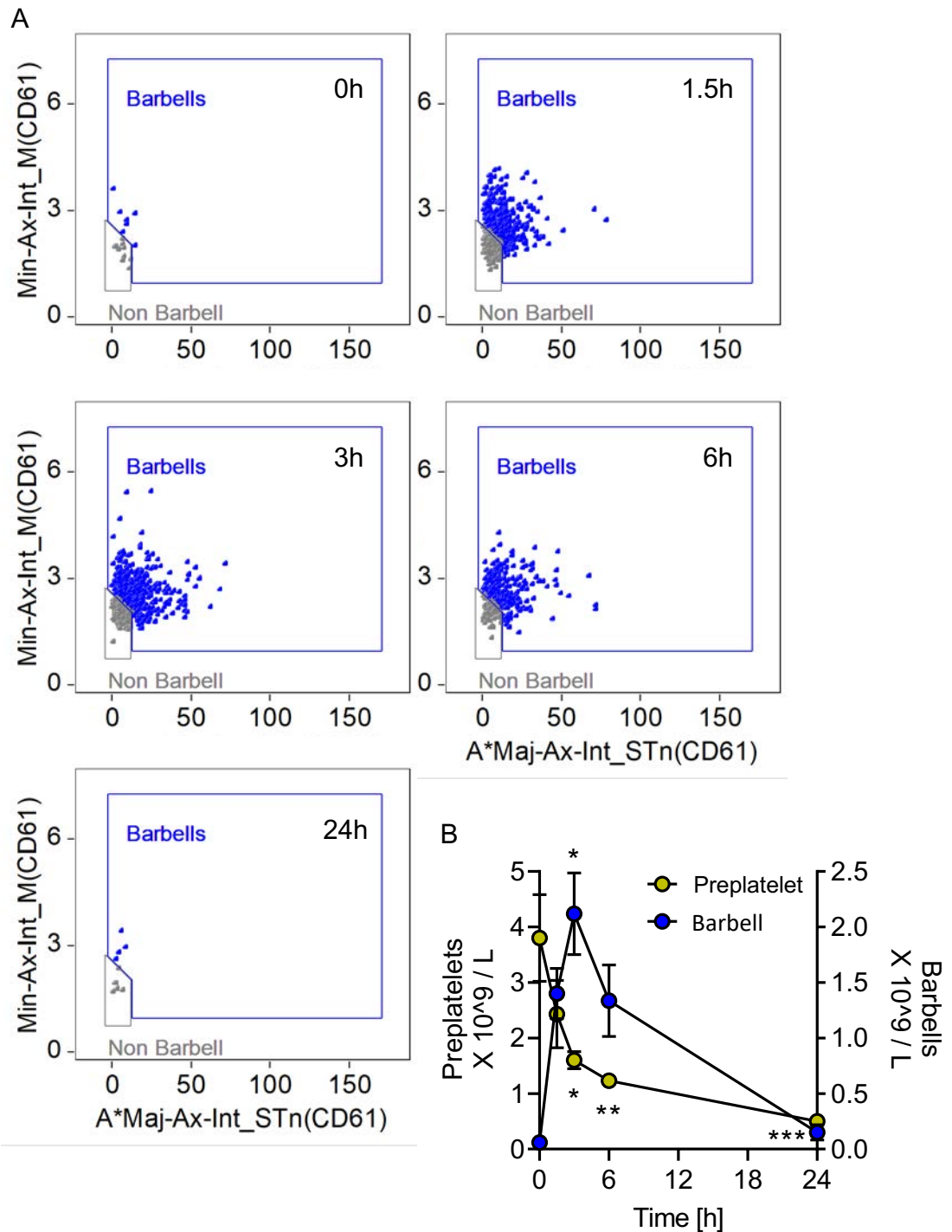


Figure 5.11: Kinetics of Preplatelet Maturation

All experiments were conducted with $100 \times 10^6/\text{mL}$ washed platelets from human control citrate blood incubated in M199 media at 37°C for a maximum of 24h. (A) Barbell platelets were visualised within the ImageStream^X IDEAS barbells gate (Figure 3.8) at 0, 1.5, 3, 6 and 24h time points ($n=10$). (B) Using ImageStream^X, preplatelets (yellow) and barbells (blue) were quantified (Figure 3.8) at 0, 1.5, 3, 6 and 24h time points ($n=7$). A*Maj-Ax-Int: Area*Major Axis Intensity; Min-Ax-Int: Minor Axis Intensity. Error bars ± 1 SD. (B) two-way ANOVA with Bonferroni multiple comparison test. Sig * <0.05 , ** <0.01 , *** 0.001 .

To track the number of preplatelet abscission events, washed platelets prepared in citrate were labelled with a cell tracking cytosolic dye (CellTrace™ yellow; 2µM). Labelled washed platelets were then resuspended in M199 media and incubated at 37°C for 24h. CellTrace™-labelled platelets were probed using ImageStream^x at 0 and 24h timepoints. Baseline measurements (0h) confirmed that 100% of platelets were CellTrace™ positive. After 24h incubation, 12% of platelets (or progeny) appeared with significantly reduced CellTrace™ labelling (Figure 5.12a) yielding on average an 11% increase in total platelet counts ($p = 0.007$; Figure 5.12b). Further interrogation of the CellTrace™ yellow fluorescence profile, suggested that preplatelets could undergo at least two rounds of fission signified by the two separate peaks (Figure 5.12c). Although not significant, the mean platelet area was reduced at 24h ($p = 0.2882$; Figure 5.12d). Together these data suggest preplatelets can undergo at least two rounds of fission to increase the platelet count and possibly regulate platelet size.

As CellTrace™ dyes bind to free amines, it was important to rule out whether the reduction in CellTrace™ MFI is simply due to the release of granules during the incubation period. To confirm this, washed platelets were labelled with CellTrace™ yellow as above and incubated at 37°C for 30 minutes with increasing concentrations of TRAP (1, 10, 100 & 1000µM ; $n=3$). Compared to control conditions, platelet granule release increased with increasing concentration of TRAP (Figure 5.13a; 1µM: 15%, 10µM: 45%, 100µM: 80% and 1000µM: 99%). However, TRAP stimulation had no effect on CellTrace™ intensity confirming reductions in platelet CellTrace™ MFI at 24h was from platelet duplication events (Figure 5.13b).

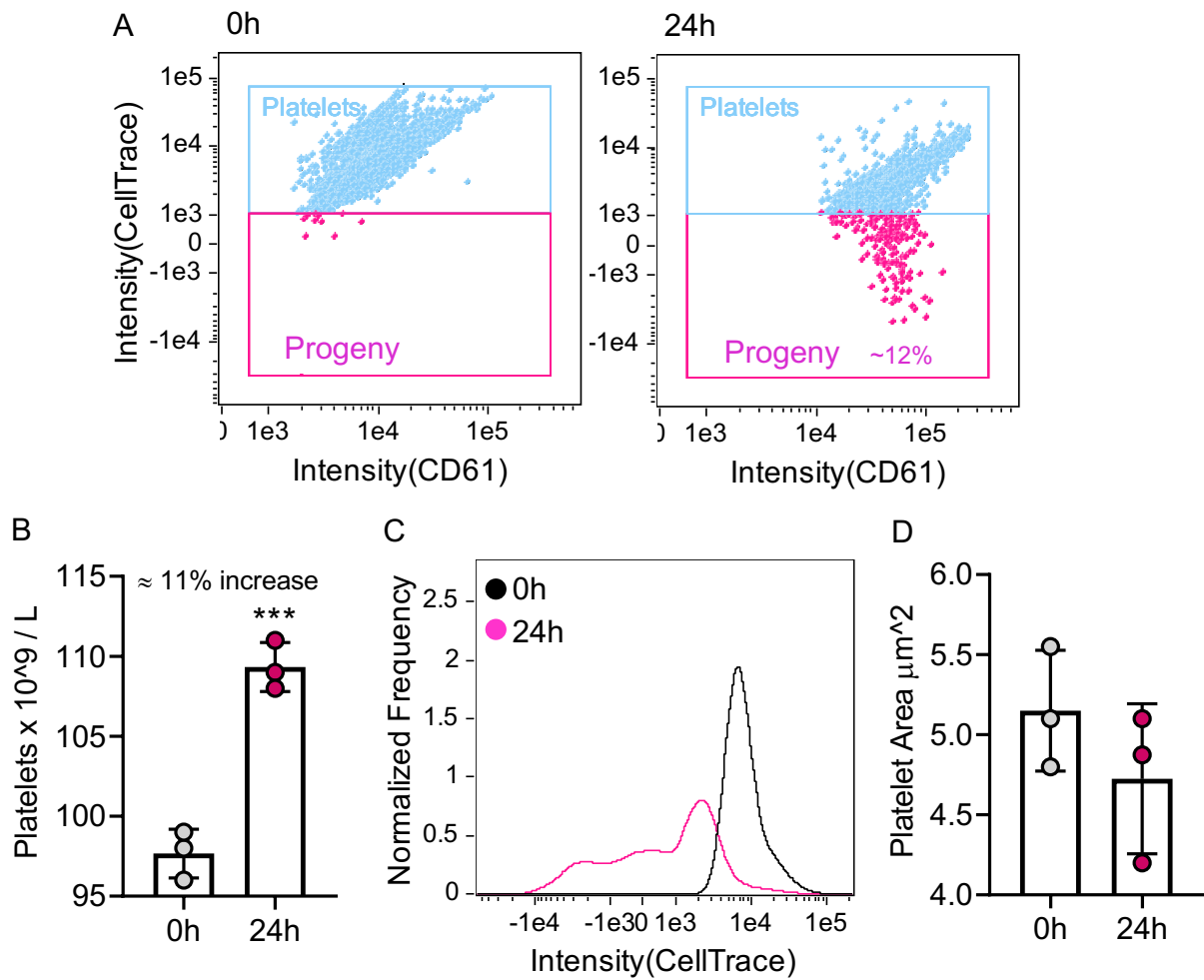


Figure 5.12: Cytosolic Labelling to Track Preplatelet Maturation Events

100x10⁶ / mL washed platelets were labelled with a CellTrace™ yellow cytosolic dye (2μM) for 30 minutes, centrifuged at 1000g with PGI₂ (10μg/mL), resuspended in a serum-free M199 media and incubated for 24h at 37°C (n=3). (A) Scatter plots demonstrating CellTrace™ labelled platelets (blue) and the appearance of a discrete population of platelets termed “Progeny” (magenta) which display a decrease in CellTrace™ yellow mean fluorescence intensity (MFI). (B) Platelet counts determined by XN1000 analyser (Sysmex) at 0h and 24h incubation times. (C) Histogram comparing total platelet CellTrace™ yellow MFI at 0h (black) and 24h (cyan). (D) Mean platelet area determined by a erode+4 mask of FITC CD61 fluorescence at 0h and 24h. Error bars +/- 1 SD. (B & D) Mann-Whitney U test. Sig. ***<0.001.

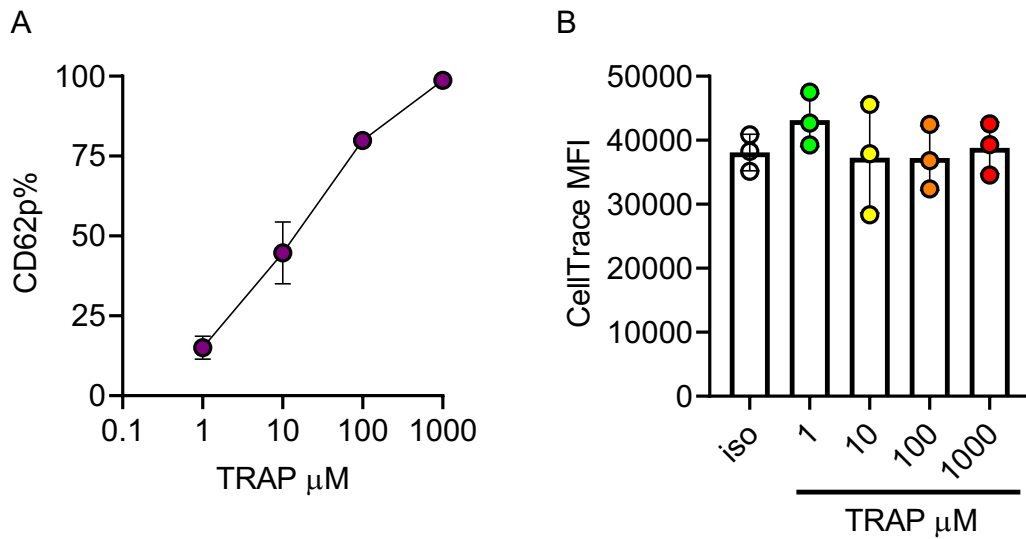


Figure 5.13: Trap-Induced Degranulation Does Not Influence CellTrace™ Labelling

Washed platelets ($100 \times 10^6/\text{mL}$) were labelled with BV421 CD62p, FITC CD61 and a CellTrace™ yellow cytosolic dye ($2\mu\text{M}$) for 30 minutes, centrifuged at $1000g$ with PGI_2 ($1\mu\text{M}$), resuspended in PBS containing increasing concentrations of TRAP (1, 10, 100 & $1000\mu\text{M}$), incubated for 30 minutes and fixed with 2% formalin ($n=3$). (A) Percentage CD62p positive platelets were determined using an isotype control by ImageStream^x (see section 2.12 “General Methods”). (B) Change in CellTrace™ median fluorescence intensity (MFI) in response to TRAP treatment was compared to isotype control (iso). Error bars ± 1 SD. (B) one way ANOVA with Dunnett’s multiple comparison test.

5.3.4 Regulating Preplatelet Maturation

Unless otherwise stated, all the experiments were performed using washed platelets ($100 \times 10^6/\text{mL}$) in M199 media (originating from citrate whole blood) incubated at 37°C . When tracking preplatelet fission events in vitro, platelets were labelled with mitotracker CMXRos ($0.5\mu\text{M}$) prior to ImageStream^x analysis to determine changes in mitotracker MFI following maturation. Thus, to determine whether preplatelet mitochondria were either split between progeny (showing a reduction in mitotracker MFI) or also underwent potential fusion or fission events (retaining signal). Interestingly, following a 24h incubation, progeny expressed a significantly higher mitotracker MFI ($p = 0.0212$; Figure 5.14a) suggesting mitochondria become hyperpolarised during preplatelet maturation. Furthermore, when incubating washed

platelets for 6h at 37°C in the presence of rotenone (an inhibitor of mitochondrial respiration; 3 μ M), barbell formation was completely inhibited ($p = <0.01$; Figure 5.14b). Evidence from our whole blood analyses in chapter 4 (Figure 4.15d), showed $\alpha_{IIb}\beta_3$ signalling potentially plays an important role in barbell formation as no barbell platelets were observed in citrate blood in the presence of eptifibatide (10 μ M). To determine the role of $\alpha_{IIb}\beta_3$ in preplatelet maturation, washed platelets were incubated for 6h in the presence of a single dose of eptifibatide (10 μ M) or a dose every 1.5h (totalling 3; as the effects of eptifibatide-based therapeutic integrilin have been shown to only last up to 2h). Figure 5.15a demonstrates that eptifibatide treatments significantly inhibit barbell formation 1.5 - 6h ($p = <0.01$). However, at 1.5, 3 and 6h incubations, observed following a single dose of eptifibatide, low levels of barbell formation was observed at 6h. One thing apparent from the ImageStream^X acquisition was eptifibatide treatment induces platelets to expose CD62p. In response to eptifibatide treatments, CD62p MFI was significantly increased by 3h incubation ($p = <0.001$; Figure 5.15b). However, coinciding with 6h barbell formation observed within the single dose of eptifibatide, CD62p MFI returned to normal.

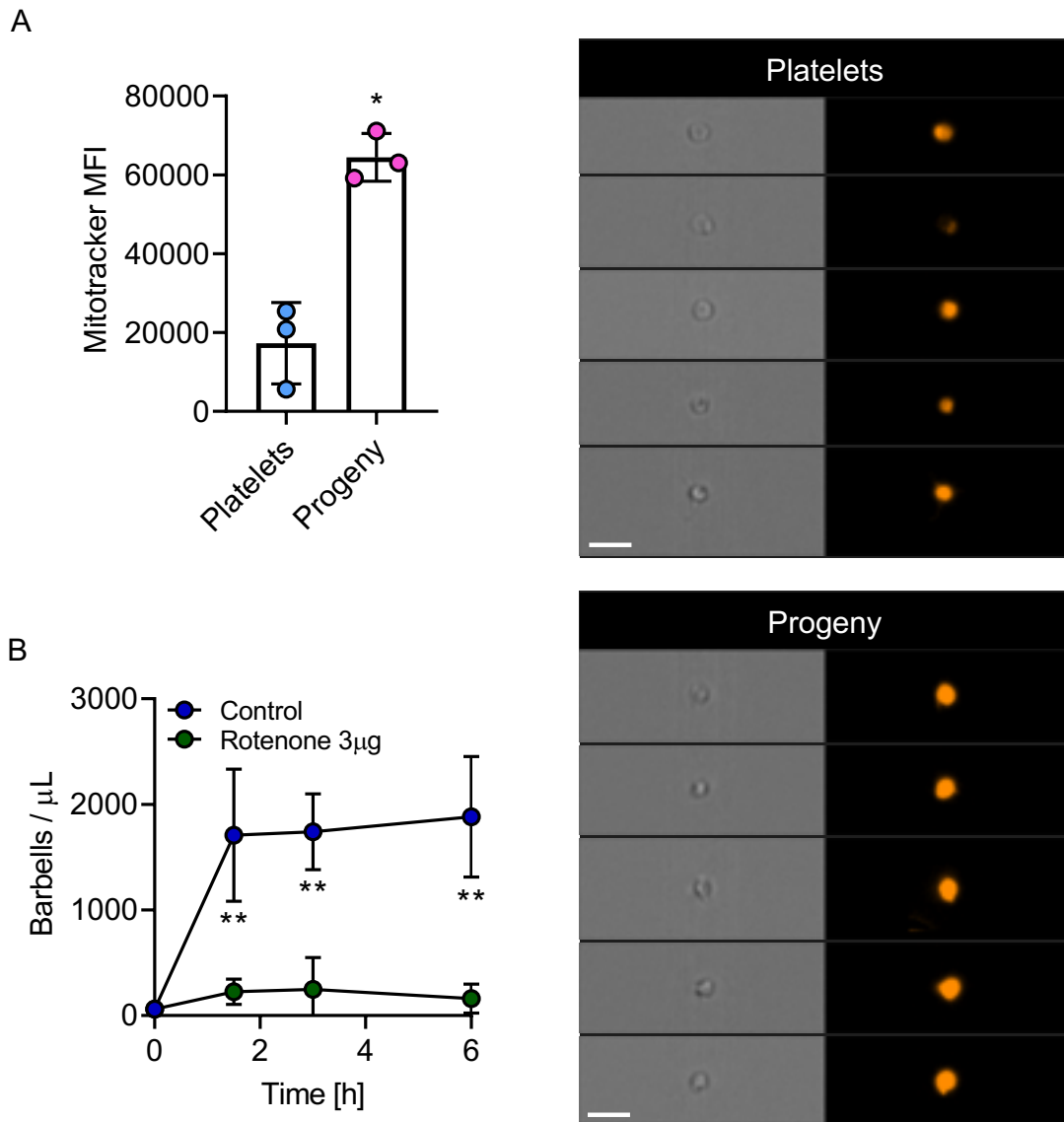


Figure 5.14: Preplatelet Barbell Conversion is Dependent on Mitochondrial Respiration

All experiments were performed using washed platelets ($100 \times 10^6/\text{mL}$) in M199 media originating from citrate whole blood. (A) Washed platelets were labelled with CellTrace™ yellow ($2\mu\text{M}$), incubated for 24h at 37°C and labelled with anti-human BV421 CD62p, FITC CD61 and AF599 mitotracker CMXRos ($0.5\mu\text{M}$; $n=3$). ImageStream^X was used to determine Mitotracker CMXRos mean fluorescence intensity (MFI) and imagery of platelets and progeny (the latter determined as platelets which displayed a significant decrease in CellTrace™ MFI). (B) washed platelets were incubated with or without rotenone ($3\mu\text{g}/\text{mL}$) for 6h and barbell platelets were measured (see Figure 3.8) at 0, 1.5, 3 and 6h using ImageStream^X ($n=5$). Scale bars $7\mu\text{m}$. Error bars ± 1 SD. (A) Wilcoxon test, (B) two-way ANOVA with Bonferroni multiple comparison test. Sig * <0.05 , ** <0.01 .

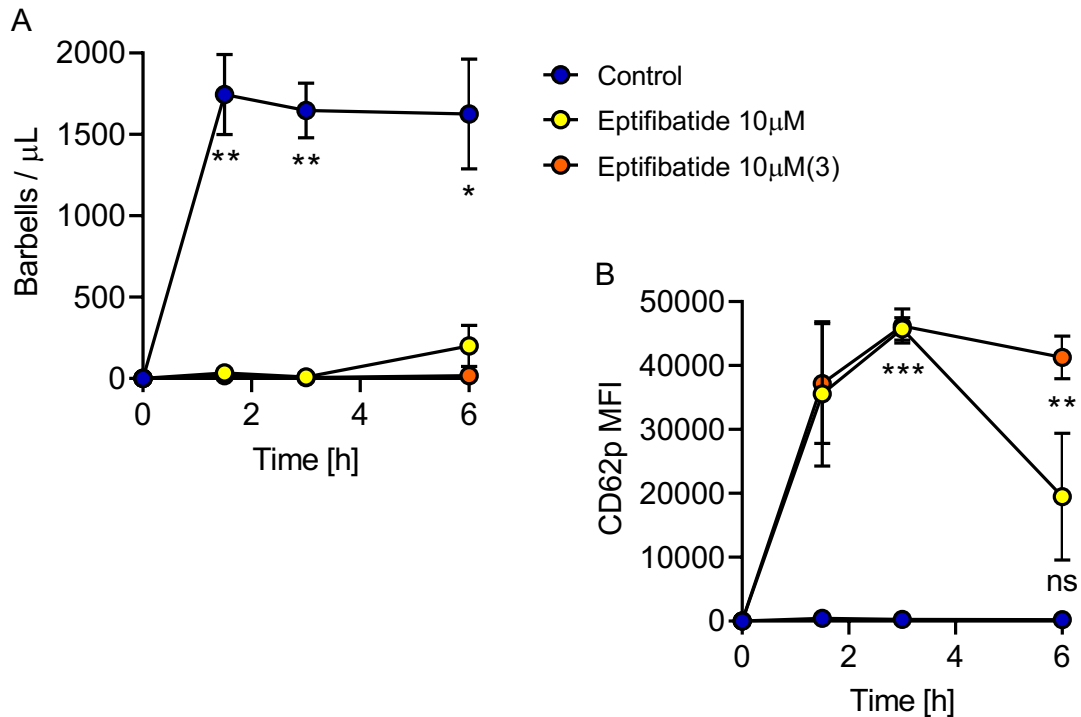


Figure 5.15: $\alpha_{IIb}\beta_3$ Signalling is Required for Barbell Conversion

All experiments were conducted using washed platelets ($100 \times 10^6/\text{mL}$), originating from citrate whole blood, in M199 media. (A) Washed platelets were incubated at 37°C for 6h without (blue) or in the presence of a single dose of eptifibatide ($10\mu\text{M}$; yellow) or the same dose every 1.5h (orange), labelled with BV421 CD62p, FITC CD61 and PE CD42b and barbells platelets were measured 0, 1.5, 3 and 6h by ImageStream^X (Figure 3.8; $n=5$). (B) CD62p exposure of following eptifibatide treatments (determined by CD62p median fluorescence intensity (MFI)) measured by ImageStream^X ($n=5$). Error bars ± 1 SD. (A & B) two-way ANOVA with Bonferroni multiple comparison test. Sig $* < 0.05$, $** < 0.01$, $*** < 0.001$.

FLNA/ $\alpha_{IIb}\beta_3$ outside-in signalling (through inhibition of small rho GTPase RhoA) have been shown to be critical for supporting normal proplatelet formation in humans [111]. Furthermore, initiation of proplatelet formation and elongation is dependent on microtubule polymerisation and sliding [163, 165]. To determine the role of cytoskeletal signalling in preplatelet/barbell formation, washed platelets were incubated for 6h in the presence of a microtubule destabilising reagent nocodazole ($10\mu\text{M}$) and two concentrations of an inhibitor of actin polymerisation, cytochalasin D (0.1 and $1\mu\text{M}$) which targets cortical or total actin respectively.

In the presence of nocodazole and cytochalasin D, barbell formation was significantly reduced ($p = 0.0174$; Figure 5.16a). Interrogation of platelet tubulin structure (determined by SiR tubulin labelling) showed under normal conditions, incubated washed platelets (6h) consisted of platelets containing both a circular-shaped marginal band and figure-of-eight-shaped barbell platelets (Figure 5.16b). In contrast, in the presence of either microtubule destabilising or actin anti-cytoskeletal drugs caused incubated platelets to remain compact. In response to nocodazole treatment, microtubules were depolymerised where no marginal band was visible and instead, the SiR tubulin signal became dense and proximally positioned (Figure 5.16b). Alternatively, platelets exposed to cytochalasin D preserved circular marginal band morphology.

TPO is the master regulator of MK differentiation and development [157, 240]. Concentrations of circulating TPO are thought to regulate the rate of platelet production across thrombocytopenic conditions [456]. Also, in vitro evidence suggests, TPO activates $\alpha_{IIb}\beta_3$ via outside-in signalling suggesting, this cytokine could potential induce preplatelet barbell transformation [457]. To determine the effect of TPO on preplatelet maturation, washed platelets were incubated in the presence of normal recombinant TPO concentrations (100pg/mL) and equivalent to that reported in AA (1ng/mL). Any changes in barbell formation was determined using ImageStream^x at 0, 1.5, 3 and 6h time points. Figure 5.17 demonstrates TPO has no effect on barbell formation.

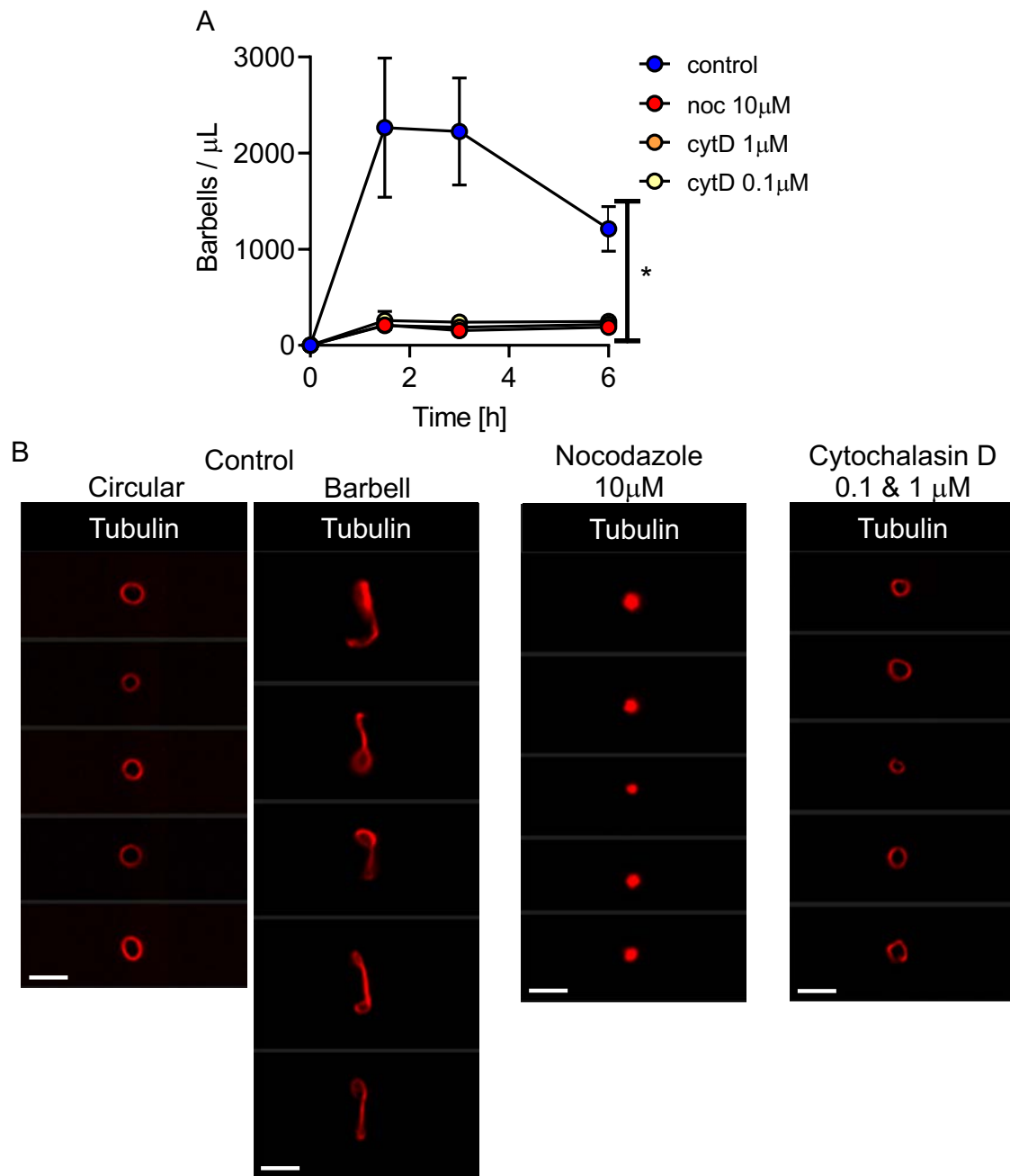


Figure 5.16: Barbell Formation Requires Normal Actin and Tubulin Polymerisation

All experiments were conducted using washed platelets (100×10^6) originating from citrate whole blood, in M199 media. (A) Washed platelets were incubated at 37°C for 6h without or in the presence of nocodazole ($10\mu\text{M}$) and cytochalasin D (0.1 & $1\mu\text{M}$) and barbell platelets were measured by ImageStream^x (Figure 3.8) and normalised with a DMSO control ($n=5$). Washed platelets were incubated for 6h and labelled with SiR tubulin ($0.4\mu\text{M}$). Platelet marginal band was depicted by SiR tubulin labelling using ImageStream^x ($n=3$; scale bar $7\mu\text{m}$). Error bars ± 1 SD. (A) two-way ANOVA. Sig. $* < 0.05$.

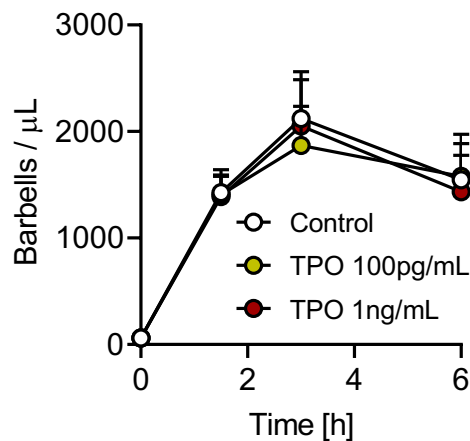


Figure 5.17: Preplatelet Maturation is Independent of TPO

All experiments were conducted using washed platelets ($100 \times 10^6/\text{mL}$) originating from citrate whole blood, in M199 media. (A) Washed platelets were incubated at 37°C for 6h without (control) or in the presence of recombinant human TPO (100pg and 1ng/mL). Barbell platelets were quantified at 0, 1.5, 3 and 6h time points using ImageStream^X (Figure 3.8). Error bars ± 1 SD. Two-way ANOVA with Bonferroni multiple comparison test.

5.4 Discussion

5.4.1 Preplatelets and Barbell Platelets are Analogous to Immature Reticulated Platelets

The findings from Chapter 4 of this thesis suggest preplatelets and their barbell derivatives are analogous to immature RP and quantitation of these maturation events was indicative of the rate of thrombopoiesis. To explore this further, we used the ImageStream^X to discriminate preplatelets and barbell platelets in healthy donors and showed (when normalised with size) these structures displayed key characteristics of newly formed platelets such as significantly increased granularity, nucleic acid content, expression of HLA I and increased labelling by mitotracker CMXRos (Figure 5.1 & 5.2). Historically, RP have been identified by CFC using size and nucleic acid content [353]. It is hypothesised RP contain both vestigial (which degrades gradually over time) and stable mRNA (used for protein synthesis), pre-packaged into platelets during

proplatelet formation [361, 458]. Biotinylation experiments have shown that RP vestigial RNA decreases rapidly within the first 24h in the circulation [5, 356, 458]. From this it is likely preplatelets should therefore be rich in vestigial RNA. Furthermore, others have shown, during barbell formation, preplatelets can increase protein synthesis and mitochondrial DNA replication [382]. Recent evidence suggests, platelets exhibit the capacity to translate mRNA into protein [167], however, it is unclear if preplatelets duplicate their entire proteome or just a subdivision during maturation. Recently, electron micrographs showed ultrastructurally, RP consist of greater amounts of α - and δ -granules compared to non-RP when normalised for size [372]. From this same study, it was concluded RP were generally larger, more haemostatically active and contained a greater number of mitochondria. When investigating the staining properties of the IPF propriety dye (FluorocellPLT®), researchers' have shown it specifically binds to platelet mitochondrial RNA [370]. The same group also reported RP have a greater number of mitochondria compared to non-RP when enhancing platelet production (determined by increases in IPF) in immunodeficient mice by injecting CD34 positive stem cells and administering eltrombopag orally for 14 days (eltro-mice) [455]. Mitochondrion health has also been implemented with platelet lifespan through the pro-survival protein Bcl-xl [226, 227, 459]. Furthermore, mice with platelets deficient in DRP-1 expression display abnormal mitochondrial morphology, circulating lifespan and reduced counts (Tugolukova; unpublished data 2017). So, it is possible, preplatelets contain a greater number of healthy mitochondria which may undergo fusion and/or fission events to maintain integrity as they mature into smaller platelets. High-resolution microscopy-based

techniques are needed to definitely show changes and localisation of DRP-1 expression during preplatelet maturation.

We also showed, mitochondrial respiration is essential for providing the energy requirements to facilitate barbell conversion (Figure 5.13b). Furthermore, following maturation, newly formed preplatelet progeny contain hyperpolarised mitochondria (Figure 13a). On average platelets contain only 5-8 mitochondria [32] yet, platelet ATP turnover is greater than other cell types which contain 100-1000 times more mitochondria [460]. Thus, platelets are more metabolically active. Interestingly, in eukaryotic cells, mitochondria become hyperpolarised during the G1/S phase transition [461]. At this point, the cell increases in size and cyclic-dependent kinase (CDK) activity promotes DNA replication. In consideration of this, it is possible during preplatelet maturation, mitochondria become fused and hyperpolarised to increase ATP production to potentially support proteome replication.

Many investigations have shown RP contain a greater granule content compared to non-RP and large platelets in general are more haemostatically active [340]. We have also demonstrated this with preplatelets and barbell platelets. RP granularity has also been linked to prothrombotic activity [462] and are associated with insufficient responses to anti-platelet drugs [463]. In addition to thrombotic function, platelets also play a key role in inflammation and immunity. For example, platelets drive T cell responses through HLA I mediated processes. Furthermore, HLA I is shed rapidly from platelets within the first 24h of circulation thus, RP contain increased HLA I expression compared to old platelets. This agrees with our findings (Figure 5.1 & 2) suggesting HLA I is also a potential marker of barbell platelets in whole blood. In contrast, preplatelets did not display a significant greater expression of HLA I. Further supporting

our findings from chapter 4 that not all circulating large platelets are preplatelets and therefore immature.

5.4.2 Preplatelet Maturation Events Represent the Rate of Thrombopoiesis in Acquired Thrombocytopenia

IPF and RP measurements assume that the proportion of circulating immature platelets is an indirect measurement of the rate of thrombopoiesis. High or low values therefore signify either enhanced BM activity or suppression respectively [362-364]. As preplatelets and their barbell derivatives showed key analogies with RP and data from chapter 4 showed the percentage of barbell platelets positively correlates with IPF% (Figure 4.14b), this suggests quantification of preplatelet maturation events in whole blood can be used as an alternative measurement to determine the rate of platelet production across thrombocytopenic conditions. Indeed, the percentage of preplatelets and barbell platelets in human ITP was significantly greater than controls with baseline measurements matching MPV and IPF% respectively (Figure 5.3a - 5.3b & 5.4). Furthermore, ex vivo barbell formation also correlated with IPF% (Figure 5.4c). Furthermore, Tubulin labelling confirmed that the frequency of barbell structures in ITP is much greater than normal, consisting of barbell platelets, elaborate proplatelet-like structures and oversized preplatelet elongated forms (Figure 5.6). Also, ImageStream^x analysis confirmed barbells were significantly larger compared to normal, originating from larger preplatelets (Figure 5.5).

In conditions such as ITP, platelet size is increased [381] and therefore, it is possible greater numbers of preplatelets enter the circulation where they may subsequently mature. Under steady state and varying degrees of thrombopoiesis, platelet size has

been shown to positively correlate with MK ploidy [335]. Thus, the higher the ploidy the larger the newly formed platelets. For example, in ITP, peripheral cues such as TPO favour increases in MK ploidy resulting in the formation of large stress platelets or preplatelets. In contrast, although BM suppression causes a reduction in all ploidy classes (preferentially the highest ploidy, 32 and 64N) [452] large platelets can still be formed. The above findings do not account for platelet shrinking or preplatelet maturation events in the circulation.

Cues such as IL-1 α and CCL5 have also been shown to enhance MK platelet production following acute cytopenic or inflammatory stress [338, 464]. IL-1 α -mediated MK rupture induces rapid release of platelets from MK, bypassing proplatelet formation whereas, CCL5 originating from platelets increase MK proplatelet formation. No studies have determined the role of IL-1 α in ITP. However, serological cytokine profiling of patients with thrombocytopenia secondary to plasmodium vivax malaria demonstrates an increase in IL-1 α [465]. In contrast, one study does show serological CCL5 is reduced in ITP and AA, positively correlating with platelet counts [466]. Thus, one can assume preplatelet formation in the BM is dependent on CCL5 and therefore the percentage of circulating preplatelets negatively correlates with serological CCL5 concentrations.

The fraction of barbell platelets and IPF in ITP varied considerably between donors (Figure 5.3 & 5.4). Although ITP is considered a model of enhanced platelet formation in response to peripheral destruction, several publications prove ITP consists of multi-causative pathogeneses. Hence, peripheral destruction can also occur simultaneously with impaired BM activity [467]. One cause of this is autoantibodies targeted against GPIb α and/or $\alpha_{IIb}\beta_3$ interfering with normal MK production and development [290, 295].

Paradoxically ITP can also be associated with thrombosis [468]. One study suggests, a thrombotic event is 1.3 times greater in patients with ITP [469] whereas, other risk factors such as age further exacerbate the risk 3-4 times. Although the cause of this is unclear, from our study, the degree of circulating barbell platelets in some individuals was striking compared to others. It is possible such a high degree of circulating large circular and elongated platelet hyperactive structures would increase the risk of spontaneous thrombus formation.

In our study, in control and ITP blood we observed that the fraction of preplatelets and barbells accurately represented platelet production and immaturity with increased BM activity (Figure 5.4c) and importantly, in response to chemotherapy-induced BM ablation, barbell platelets were almost completely absent (Figure 5.7). Interestingly both the percentage of preplatelets and IPF (two measurements dependent on size) did not differ following BM ablation. IPF and RP measurements as well as MPV often fail to distinguish between normal and conditions with BM suppression/failure and conditions with decreased production [369, 421, 470-472]. This phenomenon is thought to occur because states of low platelet counts enhance the release of RP. Also, this increase in RP production could potentially coincide with a shift in the RP lifespan. For example, in erythropoiesis, early studies suggest in response to anaemia, more reticulocytes are formed which take longer to lose their reticulum – termed the “reticulocyte shift” [473]. This same shift has been shown in mice inflicted with severe thrombocytopenia followed by dramatic thrombocytosis [474]. This could explain why very low levels of barbell platelets were still observed in the absence of BM activity.

In addition to BM failure, analysis of various macrothrombocytopaenia often gives extremely high IPF values probably caused by non-specific labelling the nucleotides of

the large platelets and is therefore unrelated to the rate of thrombopoiesis [422]. Although platelet size is an important characteristic of thrombocytopenia with peripheral destruction (e.g. ITP), it is historically reported under resting conditions that platelet size and age do not necessarily correlate [327, 343].

5.4.3 The Physiological Role of Circulating Preplatelets

Once it was established preplatelets were analogous to immature RP and the frequency of circulating barbell platelets was indicative of platelet formation in response to enhanced peripheral destruction of platelets and following BM ablation, it was important to investigate the physiological relevance of their maturation. In order to do this, it was necessary to design an in vitro assay in which preplatelet maturation events can be examined. Barbell platelet formation was first described by Schwertz and colleagues (2010), when incubating platelet concentrates, washed platelets or blood at 37°C for 6h under gentle agitation [382]. From this study it was estimated after a 6h incubation ~5% of platelets transformed into barbells. We also observed barbell platelets when incubating washed platelets for 3h under the same conditions and quantification using ImageStream^x showed barbell formation was 3 - 4 times greater than observed in incubated citrate whole blood (Figure 5.9a). As we ruled out (through dual cytosolic labelling) whether barbell structures were not an artefact of two platelets aggregating together (Figure 5.9c), this suggests, under resting conditions, barbell formation is either negatively regulated by an unknown plasma/blood component or it is simply superficially enhanced under these conditions.

As whole blood cannot be incubated at 37°C for extended periods of time due to platelet activation, haemolysis and increases in PLAs, we utilised the above washed

platelet incubation assay to determine the kinetics of preplatelet maturation over 24h. Barbell formation was observed at 1.5, 3 and 6h and was completely absent at 24h (Figure 5.10a). Furthermore, quantification of preplatelets and barbell platelets showed barbell formation was maximal at 3h and significantly decreased by 24h whereas, preplatelets significantly decreased at 3, 6 and 24h (Figure 5.10b). One other study measured preplatelets and barbell platelets in PRP incubated at 37°C for 6h [384]. Confusingly, in the above study, instead of preplatelets decreasing over time, the fraction of preplatelets increased (3.63 to 14.77%) in conjunction with the percentage of barbell platelets (0.05 to 0.78%).

In our study, preplatelets were almost absent at 24h, suggesting they had all undergone barbell transformation and subsequent fission into two smaller platelets (Figure 5.10). This was further supported with a trend showing decreases in platelet size at 24h (although non-significant; Figure 5.10d). Cell tracking experiments using CellTrace™ yellow confirmed the appearance of a new population of platelets ('progeny') with reduced fluorescence (Figure 5.11a). Remarkably, preplatelets could also undergo at least two rounds of fission signified by the two separate CellTrace™ fluorescence peaks to result in 11% more platelets (Figure 5.11b & d). In agreement with our findings, electron microscopic analysis of preplatelet ultrastructure suggests based on cell volume, the largest preplatelets contain enough cargo to generate 6 - 20 platelets [380]. However, this observation was made in mice and at a different stage of development within a murine foetal liver MK culture system and not blood. Furthermore, it is unclear whether large preplatelets undergo rounds of abscission within the lung, prior to entering post pulmonary vessels [200] and if so, how many preplatelets have already undergone or completed the maturation process?

Others have attempted to measure platelet division using CFSE labelling [451]. However, this group found it problematic distinguishing shifted CFSE clusters from the platelet population due to high variations in platelet fluorescence intensity. To overcome such limitations, they sorted a narrow fluorescence CFSE intensity slit however, exposing platelets to high shear during this process appeared to generate an unexpected high fraction of dividing platelets. Unlike this study, we used ImageStream^X to determine progeny. Differences in our findings could be explained by instrument sensitivity. However, it is still possible platelet replication events within the progeny population observed in our study were lost within the CellTraceTM cluster.

Using our washed incubation assay it was possible to explore how barbell formation is regulated. In chapter 4 we discovered barbell platelets were absent in citrated blood in the presence of eptifibatide (10 μ M; Figure 4.15b). Furthermore, microscopic interrogation of tubulin labelled PRP showed the extent in which the cytoskeleton is remodelled during barbell formation (Figure 4.11 & 4.12). As fibrinogen/ $\alpha_{IIb}\beta_3$ association is critical for initiating MK proplatelet formation in vitro [186], this suggests integrin signalling is also important for initiating preplatelet/barbell conversion. Considering this, we incubated washed platelets in the presence of eptifibatide (10 μ M) and discovered barbell formation did not occur by 6h incubation (Figure 5.14a). This level of inhibition was also apparent when destabilising tubulin and inhibiting cortical and total actin polymerisation (Figure 5.15).

For preplatelets to convert into barbells they must overcome microtubule elastic bending forces and cortical actin-myosin-spectrin compression thus, larger platelets have a larger yet thinner marginal bands to overcome such force constraints [384, 475, 476]. In agreement with this, FLNA KO mice characterised by a larger platelets with

thicker marginal bands exhibit a defect in preplatelet/barbell formation [384]. Such findings have left many questions open regarding which types of macrothrombocytopaenia have a defect in preplatelet maturation (reviewed by Thon *et al*, 2012) [385]. ITP is an acquired macrothrombocytopaenia and in subjects with $\alpha_{IIb}\beta_3$ autoantibodies MK proplatelet formation is suppressed yielding fewer and denser extensions compared to normal [477]. However, as we observed an enhancement in preplatelet/barbell formation in ITP patients (Figure 5.4b) this would suggest platelet marginal band morphology is normal and such exacerbations in barbell formation are due to increases in MPV.

Mutations in $\alpha_{IIb}\beta_3$ genes (ITGA2 and ITGB3) result in GT, a bleeding defect associated with normal platelet counts but dysfunctional platelet activation through significant reductions in $\alpha_{IIb}\beta_3$ copy number [478]. Recent findings have identified rare autosomal dominant GT macrothrombocytopaenia's where constitutive activation of $\alpha_{IIb}\beta_3$ outside-in signalling suppresses proplatelet formation yielding dense pseudopodia structures [39, 274, 435, 436, 441, 479, 480] suggesting diminished outside-in signalling is required for preplatelet barbell formation. Furthermore, FLNA/ $\alpha_{IIb}\beta_3$ outside-in signalling has been shown to be essential for supporting normal proplatelet formation in humans through inhibition of RhoA [111]. Genetic mutations in FLNA inhibits actomyosin contraction and generates a similar proplatelet morphological phenotype observed in macrothrombocytopaenia patients with dysfunctional $\alpha_{IIb}\beta_3$ signalling.

Initially we hypothesised preplatelet maturation might be regulated by circulating TPO. In many thrombocytopenic conditions serological concentrations of TPO inversely correlated with platelet production [481]. Furthermore, TPO has been shown to induce

$\alpha_{IIb}\beta_3$ outside-in signalling through G(i) and not G(q) [482]. This ultimately induces an alternative signalling pathway which is independent of phospholipase C but involving Pi3K and the small GTPase. TPO is therefore a promising candidate for initiating preplatelet maturation. However, in our study, increasing concentrations of TPO exhibited no influence on barbell formation (Figure 5.15) suggesting regulation of TPO-induced platelet production is restricted within the MK lineage only and not through preplatelet maturation.

5.5 Limitations and Future Directions

Although we show preplatelets and barbell platelets share certain analogies (including expression of platelet ageing markers and relationships between current immature platelet measurements in health and thrombocytopenia) with immature platelets and their quantitation correlate with current RP measurements (such as IPF) and therefore, could represent the rate of platelet production across conditions with varying degrees of platelet formation, further investigations are required to definitely confirm preplatelets are indeed newly formed platelets. Also, in our ITP cohort, variations in IPF as well as barbell platelet percentage, suggest differing aetiologies associated with ITP affecting platelet production. Unlike human studies, mouse models can offer rigorously controlled experimental conditions. In chapter 6, we will therefore utilise mouse models including *in vivo* biotinylation and acute platelet depletion in an attempt to definitively show preplatelets and barbells are indeed newly formed and further understand their role in response to changes in platelet counts respectively.

The 37°C incubation time used for analysing preplatelets and barbell platelets in citrate blood was 1.5h. Although this was not ideal, unfortunately, as patient recruitment often

required consultation to discuss the project before and after sampling and required transportation from the clinic to the lab it was difficult to prevent blood from cooling. However, as we observed little change in preplatelet and barbell platelet concentrations when incubating healthy control blood for 1.5h, we opted to use this timepoint to re-establish preplatelet maturation within cooled blood. Furthermore, when studying platelet viability in platelet concentrates stored for extended periods at 37°C, one group found platelet viability was normal until 3h incubation with increasing signs of apoptosis at 1 and 2 days [483]. This is the reason for the use of washed platelets for the extended incubation periods.

As in chapter 4, our patient cohort sample sizes (ITP $n=8$ and chemotherapy $n=6$) were lower than we were originally hoping for. However, although greater numbers are required to perform meaningful clinical studies, we do for the first time show preplatelet maturation represents the rate of platelet production in ITP and in the absence of platelet formation. To understand the role of preplatelet maturation in thrombocytopenia, a large-scale study is required which includes a diversity of conditions with varying aetiologies.

In our chemotherapy study we originally identified the nadir and platelet rebound phase as key time points to show changes in human preplatelet/barbell kinetics ex vivo. Unfortunately, this was not possible due to limitations imposed by the high degree of projects currently running within the ward and the unexpected premature termination of patient recruitment due to COVID19. Furthermore, the only recruited patients which did reach the rebound stage had received at least one platelet transfusion within the last 7 days. Furthermore, once the time had passed for those to be cleared from the system, platelet counts had returned to normal. Others have successfully investigated

the kinetics of RP following cytotoxic therapy to treat breast cancer [346]. These patients did not require a stem cell transplant and therefore were less likely to require intervention. Unfortunately, these patients were not available for our study.

Finally, the physiological relevance of preplatelet maturation was interpreted from an in vitro assay and not within a natural biological environment. It is unclear whether the “duplicating platelets” observed in this model by us and others previously, are indeed analogous to preplatelets within human blood. However, as preplatelets and barbells lack biomarkers for their discrimination such indications are difficult to make. Furthermore, although it was confirmed the reduction in CellTrace™ MFI (indicating cell proliferation) was not simply due to granule release, it is still unclear if this reduction was due to division of cell leakage as the platelet aged in vitro. As barbell formation is inhibited in the presence of eptifibatide, it would be interesting to determine if a reduction in CellTrace™ labelling occurs in the presence of eptifibatide. However, as the effects of eptifibatide-based therapeutic integrilin have been shown to only last up to 2h and in this thesis the eptifibatide inhibitory effect on barbell formation is lost after 3-6h (Figure 5.15a), incubated platelets would need to be treated every 2-3h to maintain barbell inhibition.

5.6 Conclusions

In this chapter we have shown preplatelets are analogous to immature RP and their maturation into barbell platelets and subsequently mature platelets increases the platelet count. This process is dependent on normal integrin and cytoskeletal signalling and is powered by mitochondrial respiration. Like RP, preplatelet maturation events represent the rate of platelet production in thrombocytopenia associated with enhance

peripheral destruction. In contrast, RP measurements have failed to discriminate between normal and BM failure blood samples. In our study, circulating barbell platelets were significantly reduced following chemotherapy-induced BM ablation suggesting quantitation of circulating barbell platelets offers a new approach to determine platelet production across thrombocytopenic conditions.

Chapter 6 - The Physiological Relevance of Preplatelet Maturation in Mice

6.1 Introduction

In Chapter 5, it was demonstrated preplatelets and their barbell derivatives share analogies with immature RP (Figure 5.1 & 5.2). Importantly, quantitation of preplatelets and barbell platelets in acquired thrombocytopenia (associated with enhanced peripheral destruction and absence of BM activity) human blood samples suggests, that quantitation of preplatelet maturation events can potentially be used as an alternative measurement of platelet immaturity and therefore platelet production to potentially aid in diagnosis and management of thrombocytopenia (Figure 5.4 - 5.8). Paradoxically, following chemotherapy-induced BM ablation, in the absence of platelet formation, the fraction of preplatelets and IPF did not differ from baseline measurements taken prior to treatment (where platelet production was normal; Figure 5.8). However, importantly, barbell platelets were all but absent under these conditions. This suggests, under steady state, not all large circulating platelets are young and therefore, preplatelets may be a subpopulation of large platelets that can form barbell platelets and undergo fission into mature platelets. Furthermore, their production is also enhanced in response to peripheral platelet destruction in ITP.

The focus of this chapter is to further explore, in murine whole blood, preplatelet maturation events under regulated conditions through the use of mouse models using either antibody-mediated platelet depletion or in vivo labelling with biotinylation. Although preplatelet maturation events have been observed by microscopy in mice, [380, 384] their dynamics have never been studied directly in blood. Furthermore, preplatelets and barbells have never been measured in mouse blood following acute, severe platelet depletion. However, in one study, researchers observed a greater number of barbells in response to rabbit anti-mouse platelet serum (RAMPS) when

incubating PRP for 6h [384]. As we observed variable results when measuring preplatelet maturation events and IPF in ITP blood, we will use platelet depletion to further study the kinetics and dynamics of immature platelets, preplatelets and barbells under precisely controlled conditions.

A key experiment to definitively determine if preplatelets and their barbell derivatives are equivalent to immature platelets is to utilise in vivo labelling using biotinylation. In vivo biotinylation studies essentially label all circulating blood cells and can be detected by flow cytometry using streptavidin conjugates and specific platelet markers (e.g. CD41 or CD61). This enables cell populations of interest (e.g. immature and mature platelets) to be tracked everyday post labelling to determine circulatory lifespan and kinetics. In vivo biotinylation in mice, in conjunction with TO labelling, has previously confirmed that large RP are indeed the youngest circulating platelets with a lifespan of only 1-1.8 days compared to a total circulating platelet lifespan of 4-5 days [5, 356]. However, under resting conditions platelet size and age have been shown to be independent [327, 343]. Using in vivo biotinylation, in combination with ImageStream^x, we will explore whether preplatelets (>3µm in diameter) and barbell platelets are equivalent to newly formed immature platelets and elucidate if preplatelets are a subpopulation of large platelets.

Therefore, the objectives of this chapter were:

1. To detect, characterise and accurately quantify preplatelets and barbell platelets in murine citrated whole blood.

2. To establish whether platelet maturation events represent the rate platelet production in response to acute thrombocytopenia.
3. To utilise in vivo biotinylation in conjunction with ImageStream^X, to definitively show whether preplatelet maturation is restricted to newly formed immature platelets.

6.2 Methods

All blood samples were either anticoagulated in 100 μ L of sterile EDTA (5mM) in PBS or 1/10 volume of 4% tri-sodium citrate. Anticoagulant/whole blood dilutions were determined by weighing a 1.5mL Eppendorf containing the anticoagulant before and after blood sampling to determine the exact dilution factors. Blood samples were taken via tail bleed for platelet counts and flow cytometry and by terminal bleed inferior caudal vena cava for preplatelet and barbell analyses. To match our human experiments, murine whole blood was incubated for 1.5h at 37°C under gentle agitation prior to performing experiments. Unless otherwise stated, following antibody labelling, samples were fixed with preheated (37°C) 2% formalin in filtered PBS for 10 minutes and diluted to 1/80 in PBS prior to ImageStream^X analysis or 1/1000 for flow cytometry. Using the same ImageStream^X methodology described in Figure 3.10, murine blood was labelled with BV421 CD62p (to exclude possible barbell-shaped and circular-shaped microaggregates) and FITC CD41a and preplatelet maturation events were discriminated and quantified using FITC CD41a fluorescence. From this, size measurements such as diameter and perimeter were determined using a FITC CD41a erode+4 mask (as described for humans) and marginal band morphology was determined by additionally labelling whole blood with 4 μ M SiR tubulin for 30 minutes

at 37°C (no formalin fixative was added when using the SiR tubulin probe). Mice RP were also measured by flow cytometry using BD Retic-Count (see section 2.10 “General Methods”). To determine the threshold for BV421 CD62p and FITC CD41a expression, isotypes control(s) (1/100) rat IgG1 λ (Clone A110-1, BD, #562604) and rat IgG1 κ (Clone RTK2071, BioLegend, #400406) respectively. TO and SiR tubulin labelling were determine using FMOs.

For platelet depletion (see section 2.15 “General Methods”), mice were injected with a polyclonal GPIb α antibody (1.5 μ g / g of body weight) IP to induce severe thrombocytopenia. Platelet counts were tracked and measured using the Pentra 60 (Horiba) haematology analyser and preplatelets and barbell platelets were measured by ImageStream^X as above.

For in vivo biotinylation (see section 2.14 “General Methods”), mice were injected IV twice (30 minutes apart) with NHS-biotin (4 mg/mL) in sterile saline. To determine the levels of platelet biotinylation, whole blood was labelled with FITC conjugated streptavidin and APC CD41a and measured by flow cytometry (see Figure 2.6 “General Methods”). Mice were bled 1h and 24h after the second biotin injection to determine the levels and kinetics of biotin labelling within platelet subpopulations including immature platelets, preplatelets and barbells using ImageStream^X. For this, whole blood was labelled with BV421 CD62p and FITC conjugated streptavidin and APC CD41a was used for preplatelet and barbell analyses instead of FITC CD41a described above and in Figure 3.10.

6.3 Results

6.3.1 Characterising Preplatelet Maturation Events in Murine Whole Blood.

To determine if preplatelet maturation events in mice were present under the same conditions used in human experiments (see chapter 4), murine blood (n=5) in EDTA or citrate was incubated for 1.5h at 37°C, labelled with BV421 CD62p and FITC CD41a, fixed in 2% formalin and preplatelets and barbell platelets were quantified using the ImageStream^X method described in Figure 3.10. The fraction of preplatelets in EDTA mouse blood was higher than citrate (~5 and ~2% respectively; Figure 6.1a) suggesting preplatelets are spheroid in EDTA. Furthermore, barbell platelets were only present in 37°C citrate blood (Figure 6.1b). Thus, all further analysis was performed at 37°C using citrated blood samples. See Appendix Figure 6.1 and 6.2 for example images of preplatelets and barbell platelets in murine citrate whole blood.

To confirm the marginal band morphology of preplatelets and barbell platelets in mice, citrated blood was incubated with BV421 CD62p, FITC CD41a and SiR tubulin (4µM) for the final 30 minutes of the 1.5h 37°C incubation. Compared to platelets, preplatelets have a normal, yet larger circular marginal band (Figure 6.2a & 6.2b). As observed in our human studies (Figure 4.11 & 4.12), murine barbell platelets also consisted of a continuous figure-of-eight marginal band containing two teardrop-shaped distal structures interconnected by a tubulin positive cytoplasmic bridge (Figure 6.2c).

Using ImageStream^X, the diameter range of preplatelets (>3µm in diameter) was determined as 3 - 5.2µm, with a mean diameter of 3.21µm (Figure 6.3a). To establish the lower size threshold which preplatelets can convert into barbells, preplatelet and barbell platelet perimeter measurements were compared. The perimeter of preplatelets ranged between 12 and 22.5µm whereas barbell platelets ranged between

12 and 62 μ m. Barbells contained a significantly higher mean perimeter than preplatelets (23 and 14 μ m respectively ($p = <0.0001$; Figure 6.3b & c).

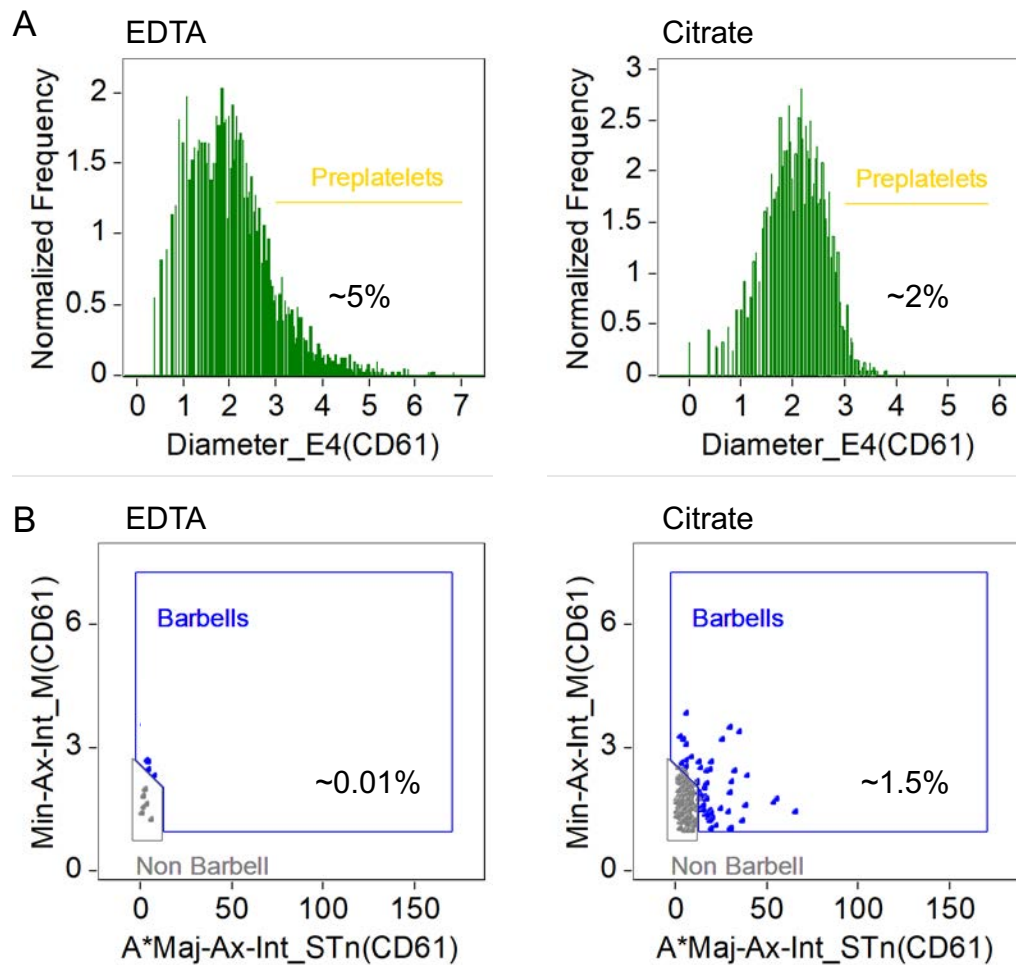


Figure 6.1: Preplatelet Maturation Events are Absent in EDTA Anticoagulated Blood

Murine blood was anticoagulated with EDTA or trisodium citrate ($n = 6$), incubated for 1.5h at 37°C, and labelled with BV421 CD62p, FITC CD41a. 10,000 CD41a platelet images were acquired using the ImageStream^X and (A) preplatelets and (B) barbell platelets were discriminated using the same methodology described in Figure 3.10 ($n=6$). E4: erode+4, Min-Ax-Int: minor axis intensity, M: morphology, A*Maj-Ax-Int: area multiplied by major axis intensity, STn: skeleton thin.

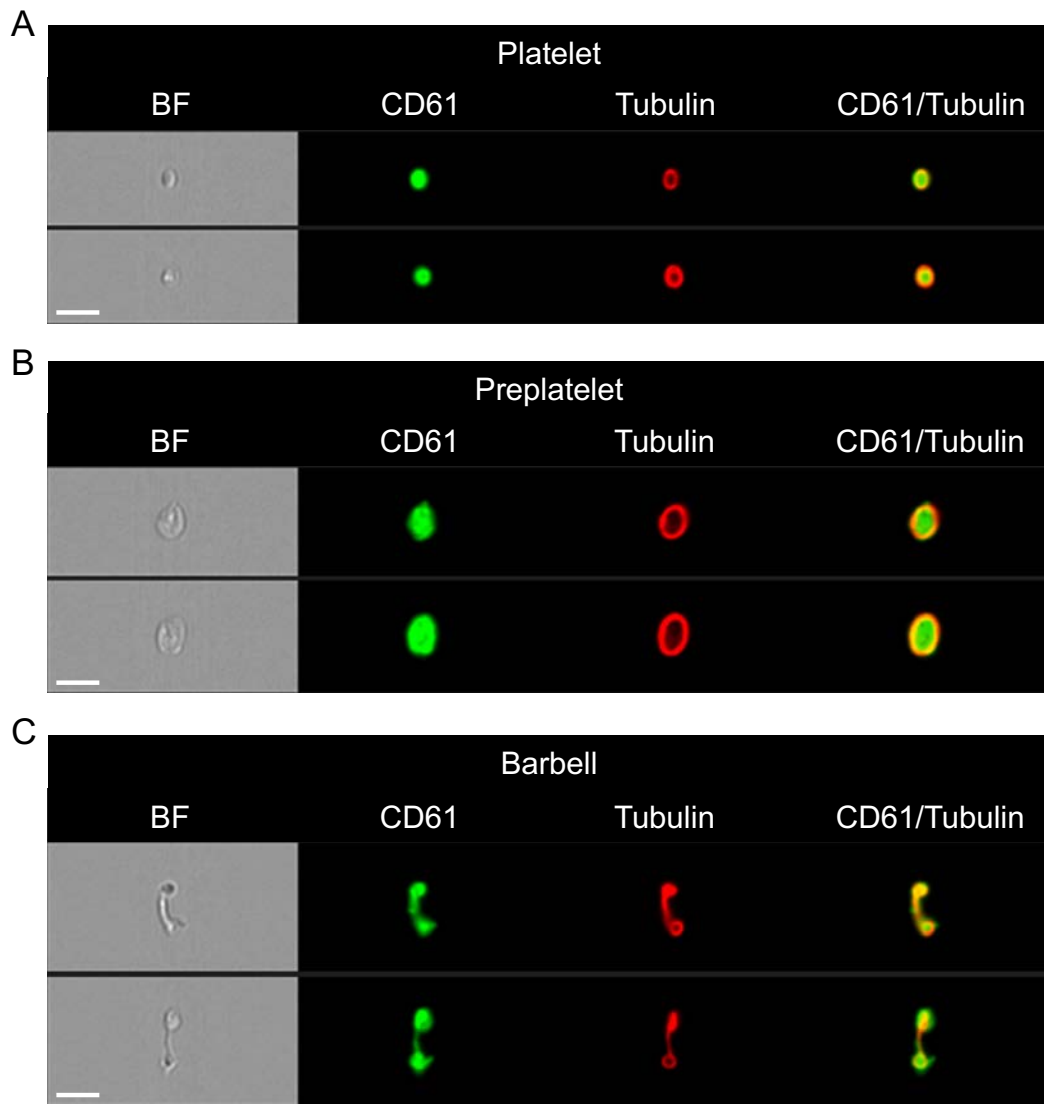


Figure 6.2: Morphology of Platelets, Preplatelets and Barbell Platelets in Murine Citrated Blood

Citrated murine blood was incubated for 1.5h at 37°C and for the final 30 minutes was labelled BV421 CD62p, FITC CD41a, and AF674 SiR tubulin (4μM; n=5). Preplatelets and barbell platelets were discriminated from 10,000 CD41a platelet images acquired using the ImageStream^x (see chapter 3, Figure 3.10). From these populations, the extracellular structure (depicted by brightfield and CD41a (green) fluorescence) and marginal band morphology (depicted by SiR tubulin (red)) was compared to resting platelets. (A) Platelets, (B) preplatelets, (C) barbell platelets. BF: brightfield, tubulin: SiR tubulin. Scale bar 7μm.

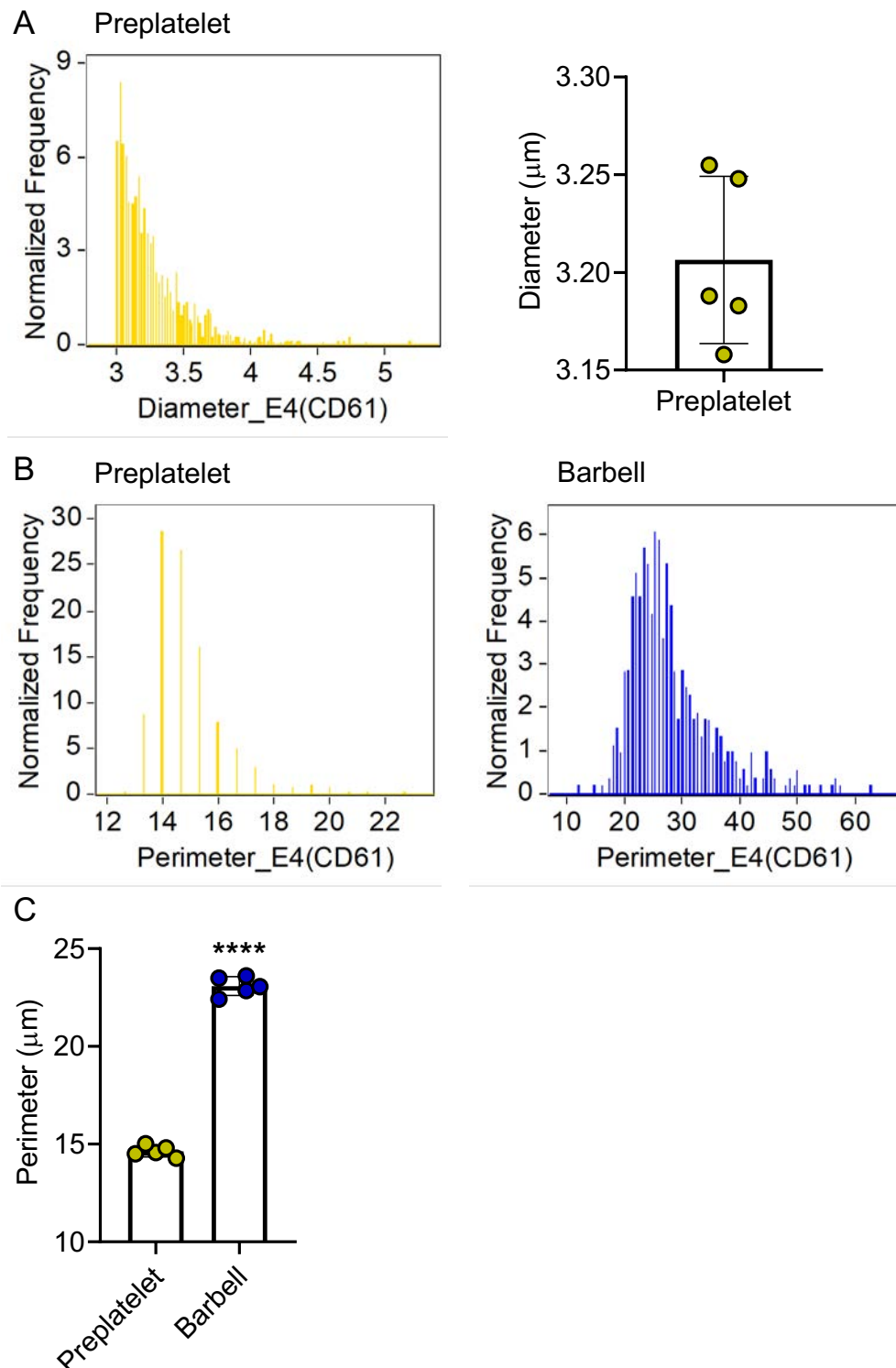


Figure 6.3: Morphometric Analysis of Preplatelet Maturation Events in Murine Citrated Blood

Citrate murine blood ($n=5$) was incubated for 1.5h at 37°C , labelled with BV421 CD62p and FITC CD41a and 10,000 CD41a platelet images were acquired using the ImageStream^X. From these images, preplatelets and barbell platelets were discriminated (Figure 3.10). Morphometric measurements of these populations were calculated using an erode+4 pixels mask (E4). These measurements include: (A) Preplatelet diameter distribution and mean and (B) preplatelet and barbell perimeter distribution and (C) mean. Error bars ± 1 SD. (C) Paired t test. **** <0.001 .

6.3.2 Determining Preplatelet Maturation in Mice

The data so far confirms that preplatelets and barbells are present within citrated mouse blood and are structurally and morphometrically similar to human blood (Figure 4.11, 4.12 & 4.13). To accurately measure these events in mouse blood, preplatelet and barbell platelets were discriminated using ImageStream^x (Figure 3.10) and compared to RP% measured by flow cytometry (see section 2.10 “General Methods”). The fraction of preplatelets and barbell platelets was similar consisting of 1.9 and 1.7% respectively of the total platelet count (Figure 6.4a). In contrast, the RP% was significantly higher than both preplatelets and barbells (7%; $p = 0.0015$ & $p = 0.0022$ respectively). Importantly, individual fractions of preplatelets and barbell platelets and combined preplatelets and barbells positively correlated with RP% ($r = 0.8509$ / $p = 0.0317$, $r = 0.8385$ / $p = 0.0385$ & $r = 0.9177$ / $p = 0.0099$ respectively; Figure 6.4b, 6.4c & 6.4d). This suggests, preplatelet maturation events in mice may represent immature platelets and represent the rate of thrombopoiesis.

To further explore, mice ($n=5$) were injected with a GPIIb α polyclonal antibody ($1.5\mu\text{g}$ / g of body weight IP) to induce acute thrombocytopenia and platelet counts were monitored over an 8-day period (see section 2.15 “General Methods”). Baseline measurements were taken at day 0. Figure 6.5a shows the kinetics of the platelet count with a rapid nadir at day 1 (~98% reduction), increasing to $\sim 250 \times 10^9/\text{L}$ at day 5 (~25% recovery) and near normal by day 8. Compared to day 0 (resting state production), both platelet MPV and RP% were significantly increased at day 5 ($p = <0.0001$ & $p = 0.0015$ respectively; Figure 6.5c & d) confirming that the platelet population consists of large numbers of newly formed immature platelets.

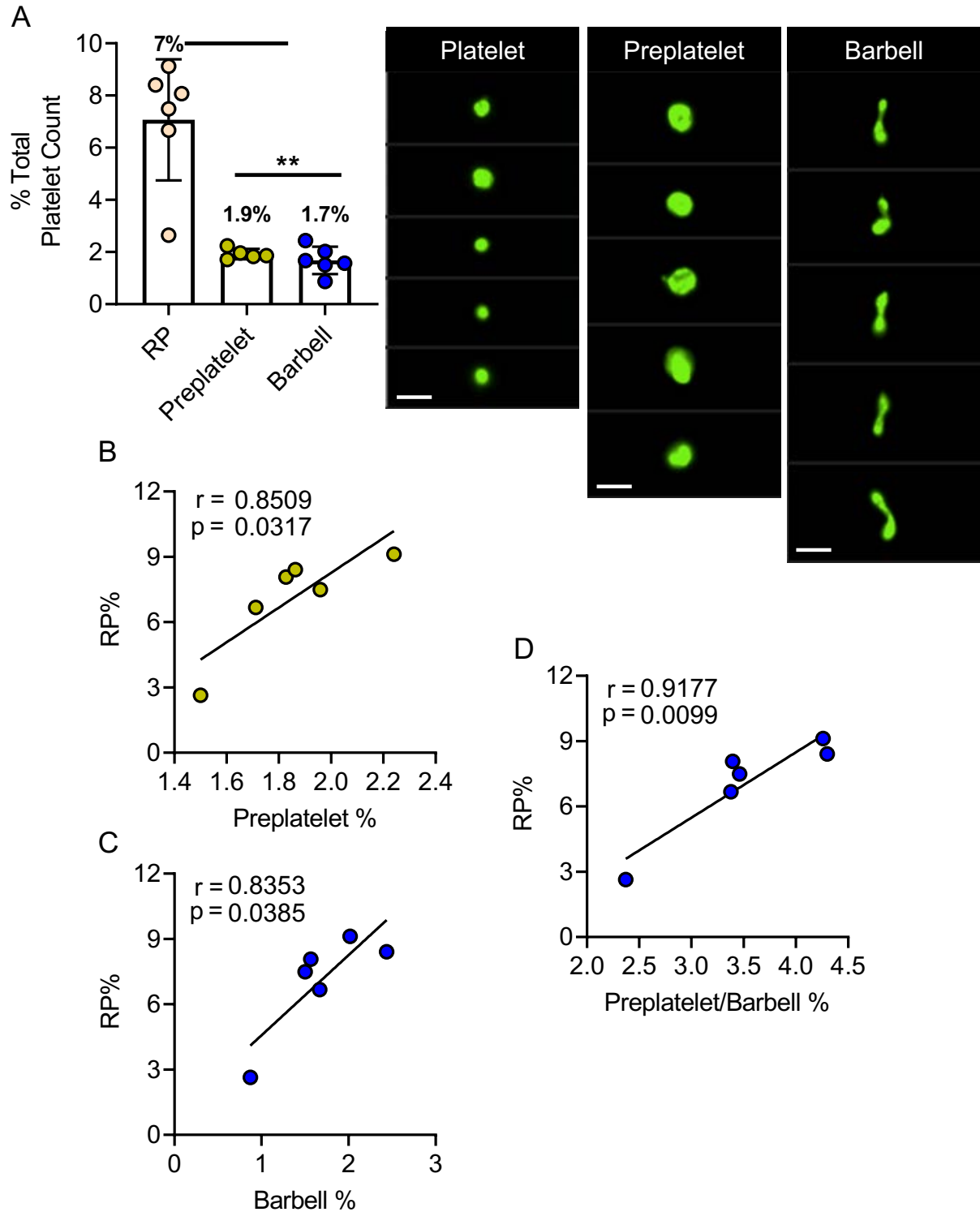


Figure 6.4: Quantifying Preplatelet Maturation Events in Murine Blood

Murine citrate blood (n=6) was incubated for 1.5h at 37°C and labelled with BV421 CD62p and FITC CD41a. (A) preplatelet and barbell platelets were quantified by ImageStream^x from 10,000 CD41a platelet images (Figure 3.10) and compared to the reticulated platelet% (RP%) measured by flow cytometry. Examples of CD41a positive platelet, preplatelet and barbell platelets imaged by ImageStream^x (x60 lens; scale bar 7 μm). Correlations between (B) preplatelet% (yellow), (C) barbell platelet% (blue) or (D) combined (green) with RP%. (A) one way ANOVA with Tukey multiple comparison test; (B-D) Pearson's Coefficient. Error bars +/- 1 SD. Sig. * < 0.05, ** < 0.01.

To establish the effect of thrombocytopenia on circulating preplatelet maturation events, preplatelets and barbell platelets were measured using ImageStream^x (Figure 3.10) on day 0- and 5-days following depletion. The fraction of preplatelets and barbell platelets was significantly increased by day 5 (~2 to ~5%, $p = 0.0033$ and ~1 to ~4%, $p = 0.0003$ respectively; Figure 6.6a & 6.6b). In fact, in some cases, preplatelets and barbells were between 4- and 5-fold greater. Furthermore, both individual and combined fractions of preplatelets and barbells, positively correlated with RP% ($r=0.9724 / p<0.0001$, $r=0.95 / p<0.0001$ and $r = 0.9629 / p = <0.0001$ respectively; Figure 6.6c, 6.6d & 6.6e). In addition, preplatelets and barbell platelets observed on day 5 were significantly larger than those on day 0 ($p = 0.0003$ & $p = 0.0002$ respectively; Figure 6.7). This suggests, in response to severe thrombocytopenia, MK produce a greater number of preplatelets which are larger than those under conditions of steady state production.

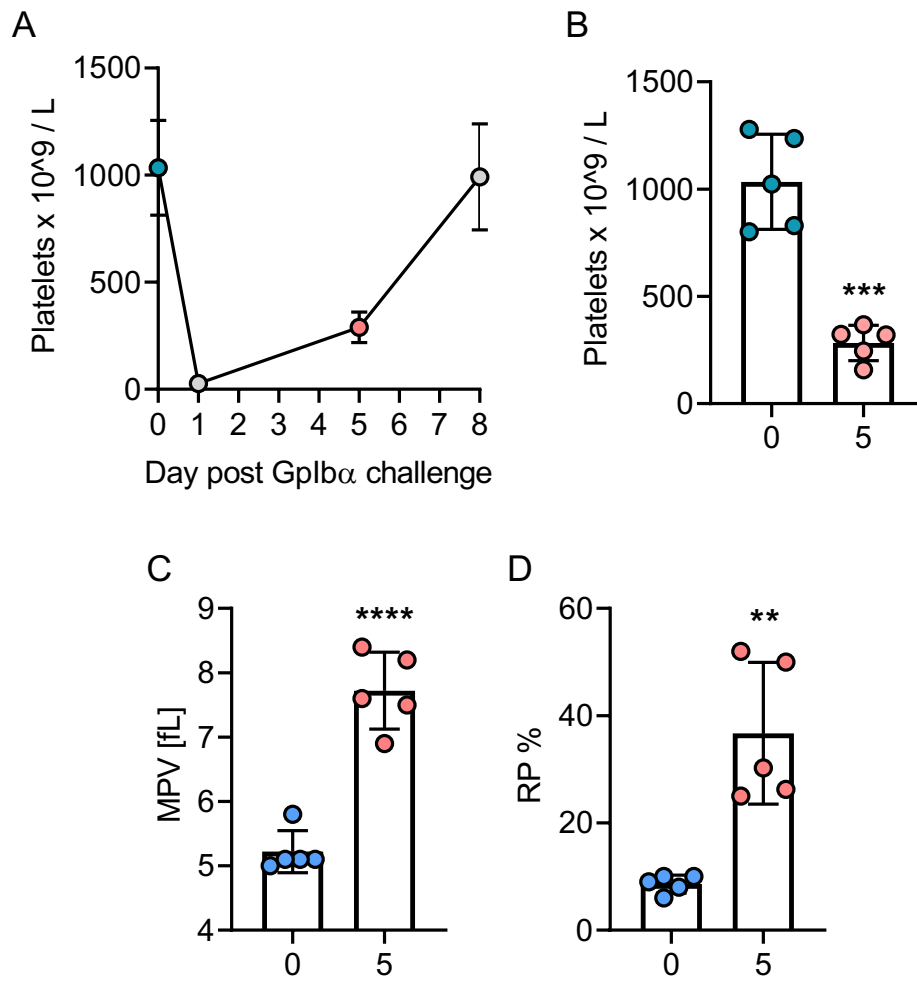


Figure 6.5: Effects of Anti-GPIIb/IIIa Treatment on Platelet counts, MPV and Reticulated Platelets

Mice (n=5) were challenged with a GPIIb/IIIa polyclonal antibody (1.5µg / g of body weight) IP on day 0 and (A) platelet counts were measured by the ABX Pentra 60 (Horiba) haematology analyser on days 0, 1, 5 and 8. Comparison of platelet count/µL of blood (B), MPV (determined by ABX Pentra 60) (C) and RP% (D) (determined by flow cytometry) at day 0 and day 5. Error bars +/- 1 SD. (B-D) Unpaired t-test. Sig. **<0.01, ***<0.001, ****<0.0001. MPV: Mean Platelet Volume; RP: Reticulated Platelet.

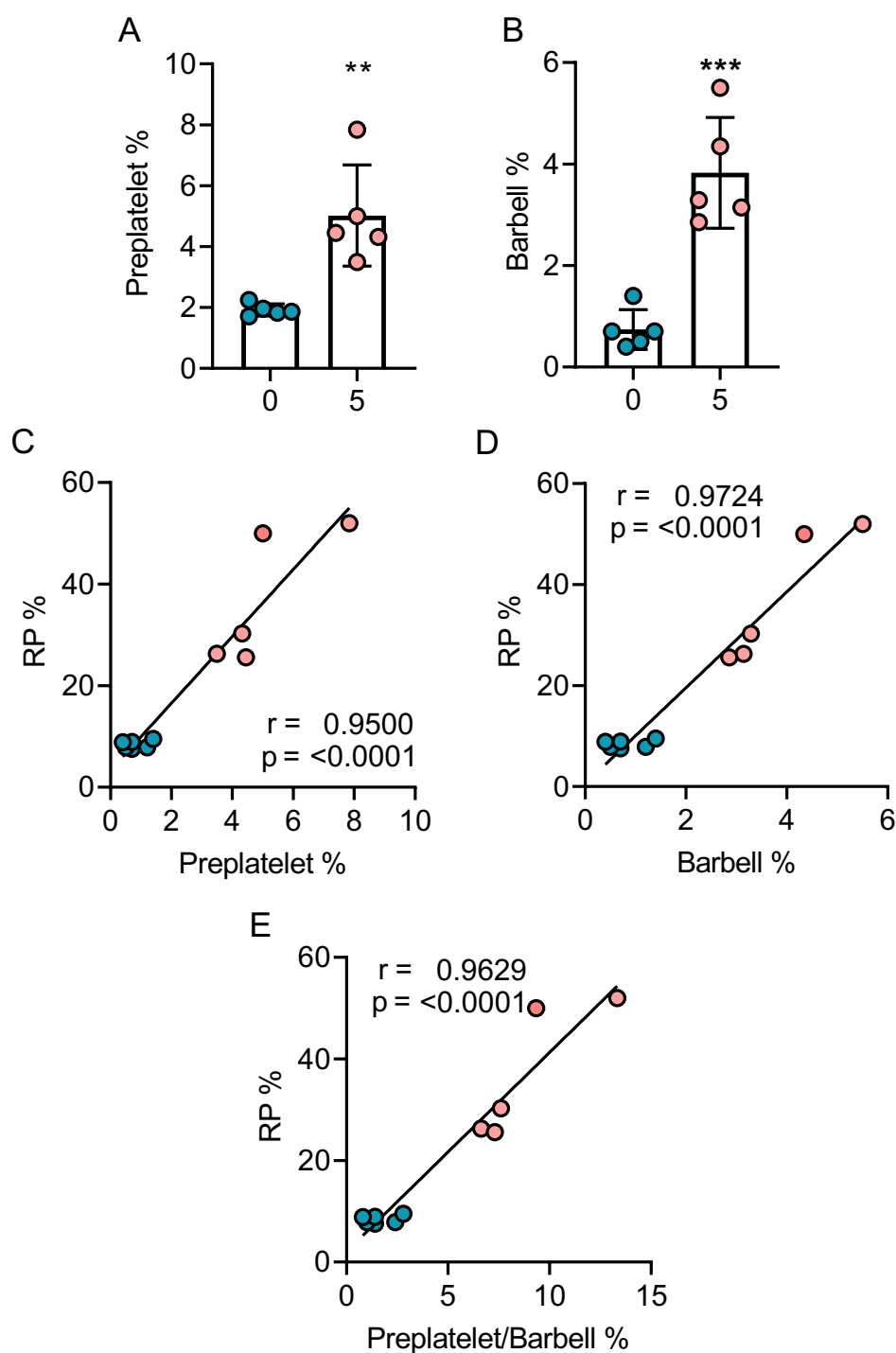


Figure 6.6: Preplatelet Maturation Events Significantly Increase in Response to Acute Thrombocytopenia

Murine citrated blood samples ($n=5$) were taken on day 0 (resting state production; blue) and 5 (recovery phase; pink) following GPIb α -mediated platelet depletion and incubated for 1.5h at 37°C. Blood was labelled with BV421 CD62p and FITC CD61. (A) preplatelet and (B) barbell platelets were quantified from 10,000 CD41a platelet images acquired by the ImageStream^x (Figure 3.10). Correlations between (C) preplatelets, (D) barbell platelets or (E) combined fractions with RP%. Error bars $\pm$ 1 SD. (A&B) Unpaired t-test; (C-E) Pearson's Correlation Coefficient. Sig. ** <0.01 , *** <0.001 , **** <0.0001 . RP: Reticulated Platelet.

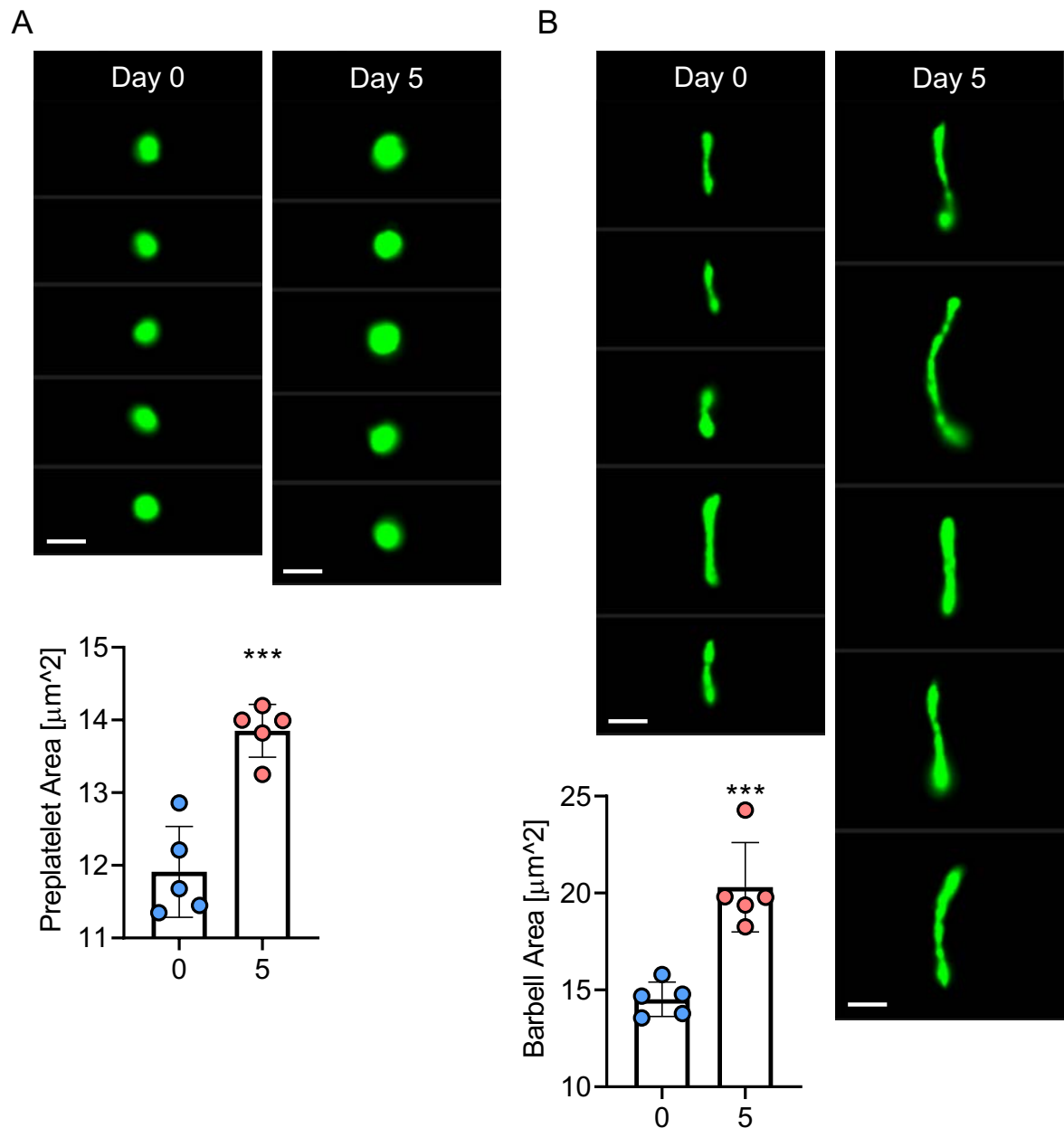


Figure 6.7: Barbell Platelets are Significantly Larger Day 5 Following Platelet Depletion
 Murine citrated blood samples (n=5) were taken on day 0 (resting state production; blue) and 5 (recovery phase; pink) following anti-GPIb α -mediated platelet depletion and incubated for 1.5h at 37°C. Blood was labelled with BV421 CD62p and FITC CD61. (A) Preplatelets and (B) barbell platelets were discriminated by ImageStream^x (Figure 3.10) Morphometric measurements were determined using an erode+4 mask of CD41a fluorescence. (A) Comparison of preplatelet diameter and (B) barbell platelet area at day 0 and 5. Preplatelet and barbell images are depicted by FITC CD41a fluorescence (scale bar 7 μm ; x60 lens). Error bars +/- 1 SD. Unpaired t-test. Sig. ***<0.001.

6.3.3 Barbell Platelets Originate from Newly Formed Immature Platelets

Both the human and mouse data suggest preplatelet formation is enhanced in conditions of increased platelet production. Furthermore, both preplatelets and barbells seem to be equivalent to immature RP. However, data from our chemotherapy patient cohort (Figure 5.9) suggests (in the absence of BM production at least) that not all large platelets are equivalent to preplatelets. To further explore these relationships under resting conditions, in vivo biotinylation in conjunction with ImageStream^X has been utilised to determine the size and morphology of newly released platelets in WT mice (n=4).

Immediately (1h) following biotinylation (day 0 time point), FITC conjugated streptavidin/biotin binding (determined by flow cytometry) shows >98% of APC CD41a positive platelets were labelled with biotin (orange gate). On day 1 (24h later; Figure 6.8a), there was a significant decrease ($p = 0.0087$) in the total biotin positive population with approximately 10% biotin negative platelets (black gate; Figure 6.8b) representing the immature platelets that have been released into circulation since the final biotin injection (24h prior).

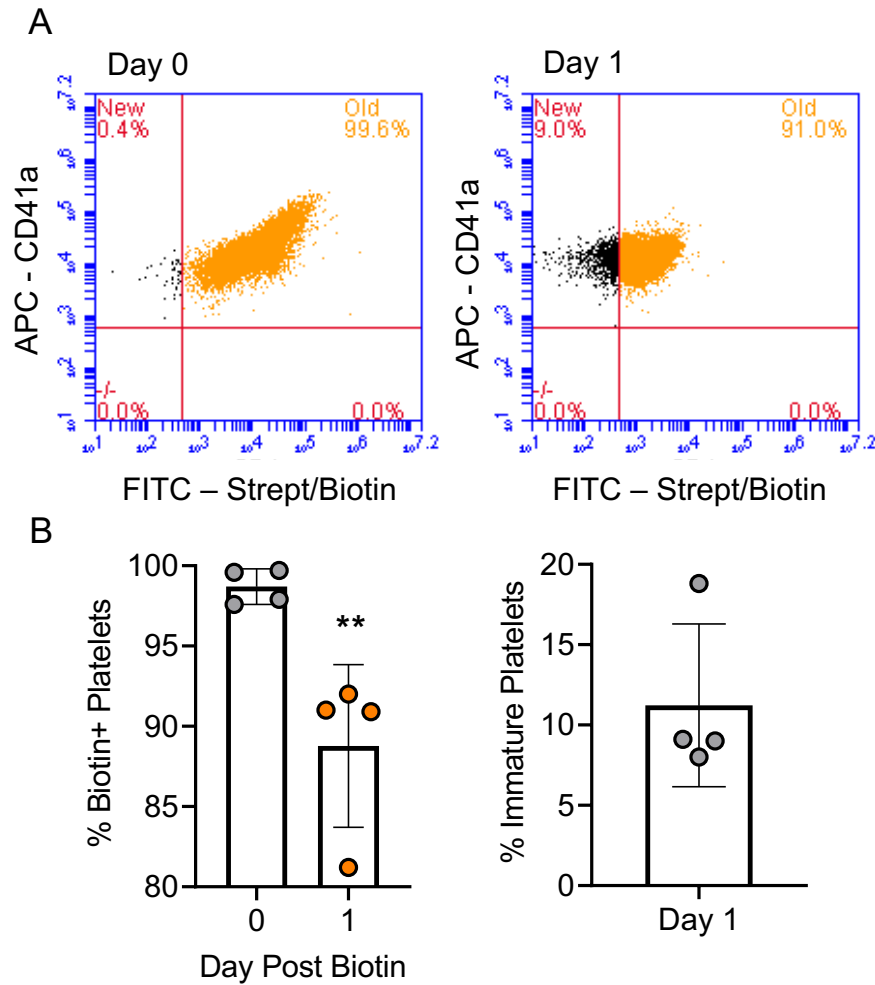


Figure 6.8: In Vivo Biotinylation of Platelets

WT mice (n=4) were injected twice (30 minutes apart) IV with NHS-Biotin (4mg/mL). Mice were bled via tail bleed (day 0) and terminal bleed (day 1) into citrate anticoagulant. Blood samples were incubated for 1.5h at 37°C and labelled with APC CD41a and FITC conjugated streptavidin. Biotinylation was measured by flow cytometry (Accuri™ C6) ; (A) day 0 and 1 (biotin positive and negative CD41a platelets are illustrated by the orange and black gates respectively) . (B) Percentage biotin positive platelets on day 0 and 1 and mean percentage of immature platelets (biotin negative) on day 1. Error bars +/- 1 SD. (B) unpaired t-test. Sig. **<0.01. Strept/Biotin: streptavidin/biotin labelling.

To compare the size and morphology of immature (black) and mature (orange) platelets, mice were bled 24h post biotinylation into citrate and blood was incubated for 1.5h at 37°C prior to ImageStream^X acquisition. For these experiments, APC CD41a (instead of FITC CD41a) was used for preplatelet and barbell discrimination (Figure 3.10) and FITC streptavidin for biotin labelling. 10,000 APC CD41a positive platelet images were acquired and biotin positive and negative platelets were determined using MFI. Images taken by the ImageStream^X (x60 lens) showed, biotin negative (immature) platelets consisted of platelets, preplatelets and barbells, whereas biotin positive (mature) platelets consisted of platelets and preplatelets (Figure 6.9a & 6.9b respectively).

Interrogation of platelet images using BF imaging and APC CD41a and FITC streptavidin/biotin fluorescence shows both the platelet and preplatelet populations consisted of a similar degree of biotinylation. In contrast, barbell platelets were all negative for biotinylation ($p = 0.0022$) suggesting they originated from large newly formed platelets. Together these findings suggest not all large platelets are immature and preplatelets are a subpopulation of large platelets. Additional images of preplatelets and barbells (including biotinylation fluorescence) can be viewed in Appendix Figure 6.3 & 6.4.

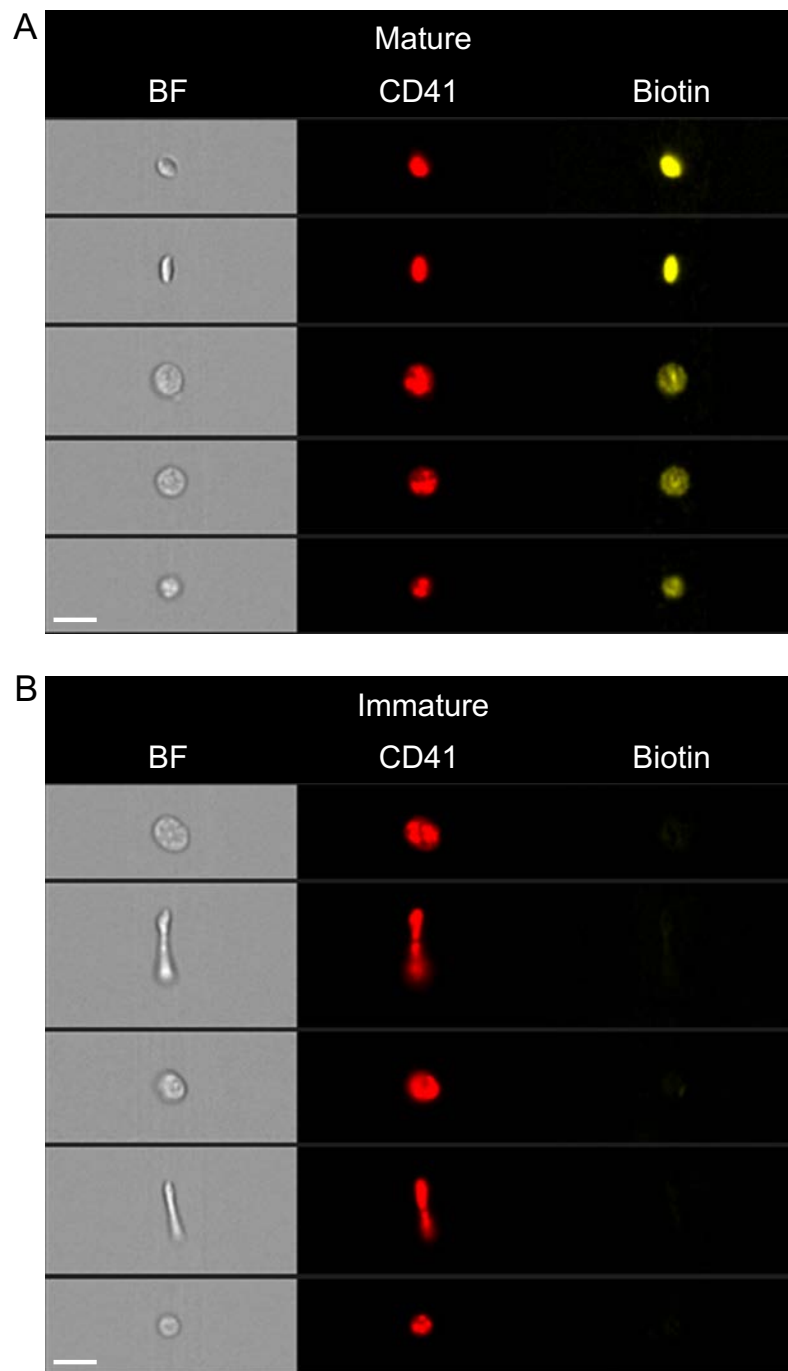


Figure 6.9: In Vivo Biotinylation Analysis by ImageStream^X

On day 1 (24h) following biotinylation, mice were bled via terminal bleed into citrate and blood samples were incubated for 1.5h at 37°C, labelled with BV421 CD62p, APC CD41a and FITC streptavidin (to label biotin) and 10,000 CD41a positive platelet images were acquired using the ImageStream^X (x60 lens). A fluorescence morphology mask was used to gate CD41a / biotin negative (immature) and positive (mature) images. Examples of (A) mature and (B) immature platelets are shown using brightfield (BF) and CD41(red) / biotin (yellow) fluorescence imaging. Scale bar 7µm. M: morphology. Figure is representative of a single experiment (n=4).

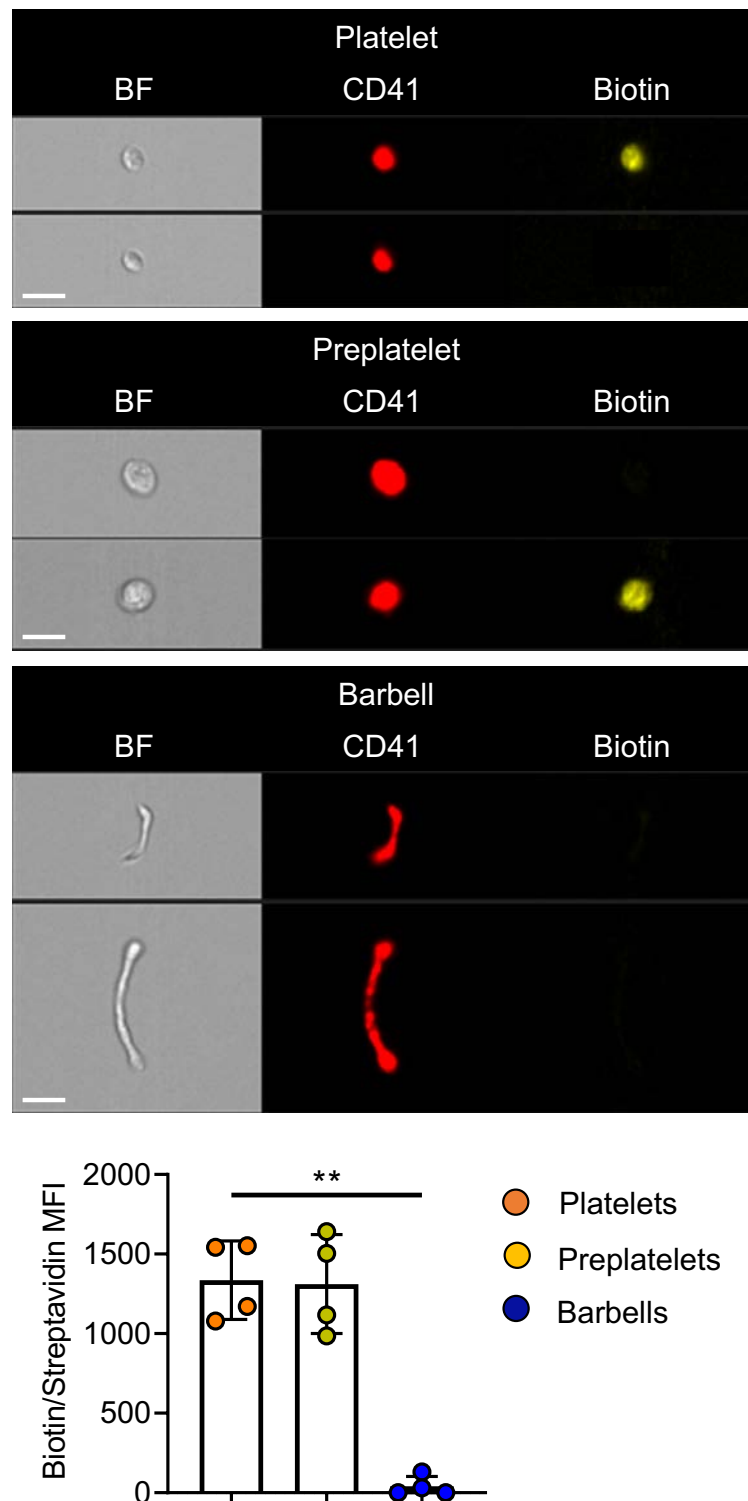


Figure 6.10: Circulating Barbell Platelets Originate from Newly Formed Platelets

Using the same day 1 blood preparation described in Figure 6.9, Preplatelets and barbell platelets were determined by ImageStream^X (Figure 3.10) from 10,000 APC CD41a positive platelet images instead of FITC CD41a (n=4). Biotinylation of preplatelets and barbell platelets was compared to normal platelets (circular platelet images <3 μ m in diameter). Images of platelets, preplatelets and barbells were depicted by ImageStream^X (x60 lens) using brightfield (BF) imaging and CD41a/biotin fluorescence (scale bar 7 μ m). Error bars +/- 1 SD. One-way ANOVA with Dunnett's multiple comparison test. Sig. <0.01.

As the platelet and preplatelet populations contained images with varied biotinylation, ranging from negative to highly positive, we use ImageStream^x to further characterise the size distributions of the total immature and mature populations to determine whether immature platelets were indeed all larger than mature platelets. The size of mature and immature platelets was determined using the area of APC CD41a fluorescence morphology. Although the minimum platelet sizes of mature and immature platelets were not significantly different (Figure 6.11a), the maximum platelet area of immature platelets was significantly higher ($p = <0.0001$). The mean platelet area of immature platelets was also significantly greater than mature ($p = 0.0414$). This suggests mature and immature platelets contain both small and large platelets, yet immature platelets are on average larger.

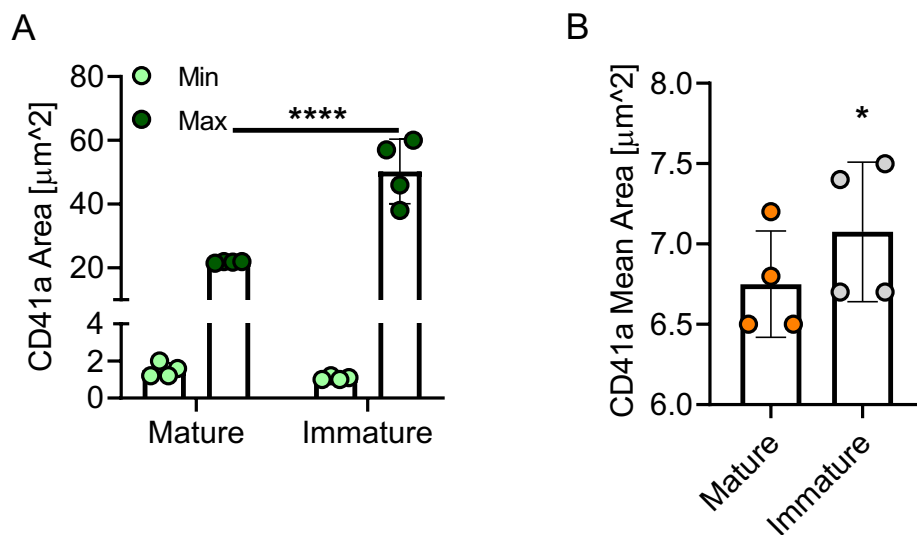


Figure 6.11: Size Measurements of Immature and Mature Platelets

Citrate murine blood was prepared and labelled as in Figure 6.9. The (A) minimum (Min), maximum (Max) and (B) mean area of biotin negative (immature) and positive (mature) platelets ($n=4$) was measured by ImageStreamX at day 1 post biotinylation (see Figure 6.9). Error bars ± 1 SD. (A) two-way ANOVA with Sidak's multiple comparison test; (B) paired t test. Sig. $* < 0.05$, $*** < 0.001$.

To further explore the size distribution of mature and immature platelets, the platelet area of these populations was plotted as a histogram. Vertical regions (or “bins”) were manually selected along the area axis to determine cell morphology. Immature platelets consisted of elongated barbell platelets and circular platelets $< 3\mu\text{m}$ in diameter (examples of platelet morphology is depicted by BF imaging and arrows show the positional values along the size distribution; Figure 6.12). In contrast, no elongated structures were present in the mature population however, circular platelets ranged from small to large containing preplatelets ($>3\mu\text{m}$ in diameter) situated at the upper end of the size distribution (Figure 6.13). Interestingly, $\sim 50\%$ of preplatelets (platelet $>3\mu\text{m}$ in diameter) were situated within the mature and immature populations (data not shown). Thus, suggesting not all large platelets are immature.

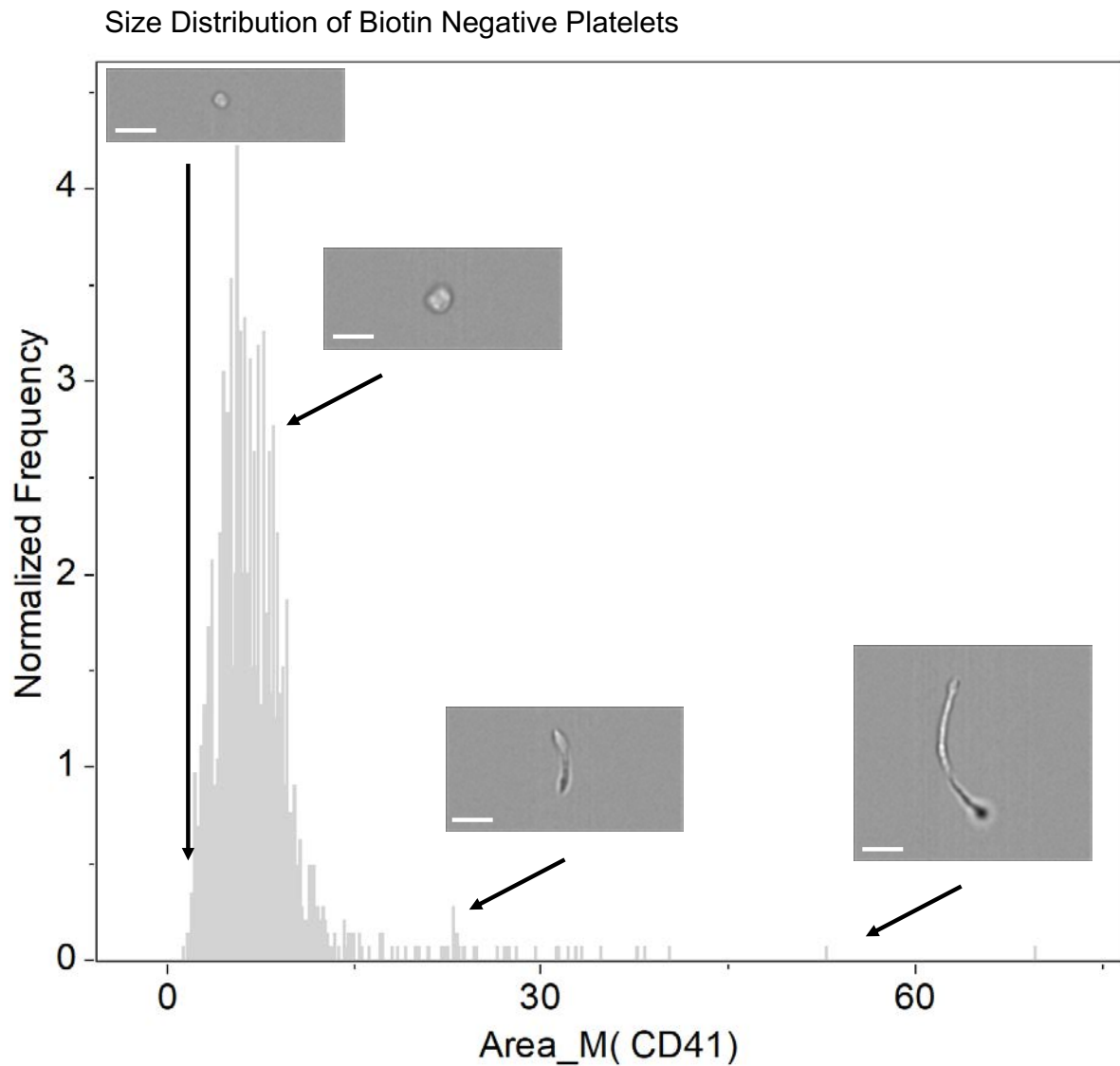


Figure 6.12: Size Distribution of Biotin Negative Platelets

A histogram to show the size distribution (determined by area of APC CD41a fluorescence morphology (M) of biotin negative (immature) platelets (n=4) at day 1 post biotinylation was measured by ImageStream^X (see Figure 6.9). Examples of CD41a positive platelet images (determined by Brightfield imaging) are also included to demonstrate their approximate positional value (determined by black arrows) along the Area_M(CD41) axis. Scale bar 7 μ m.

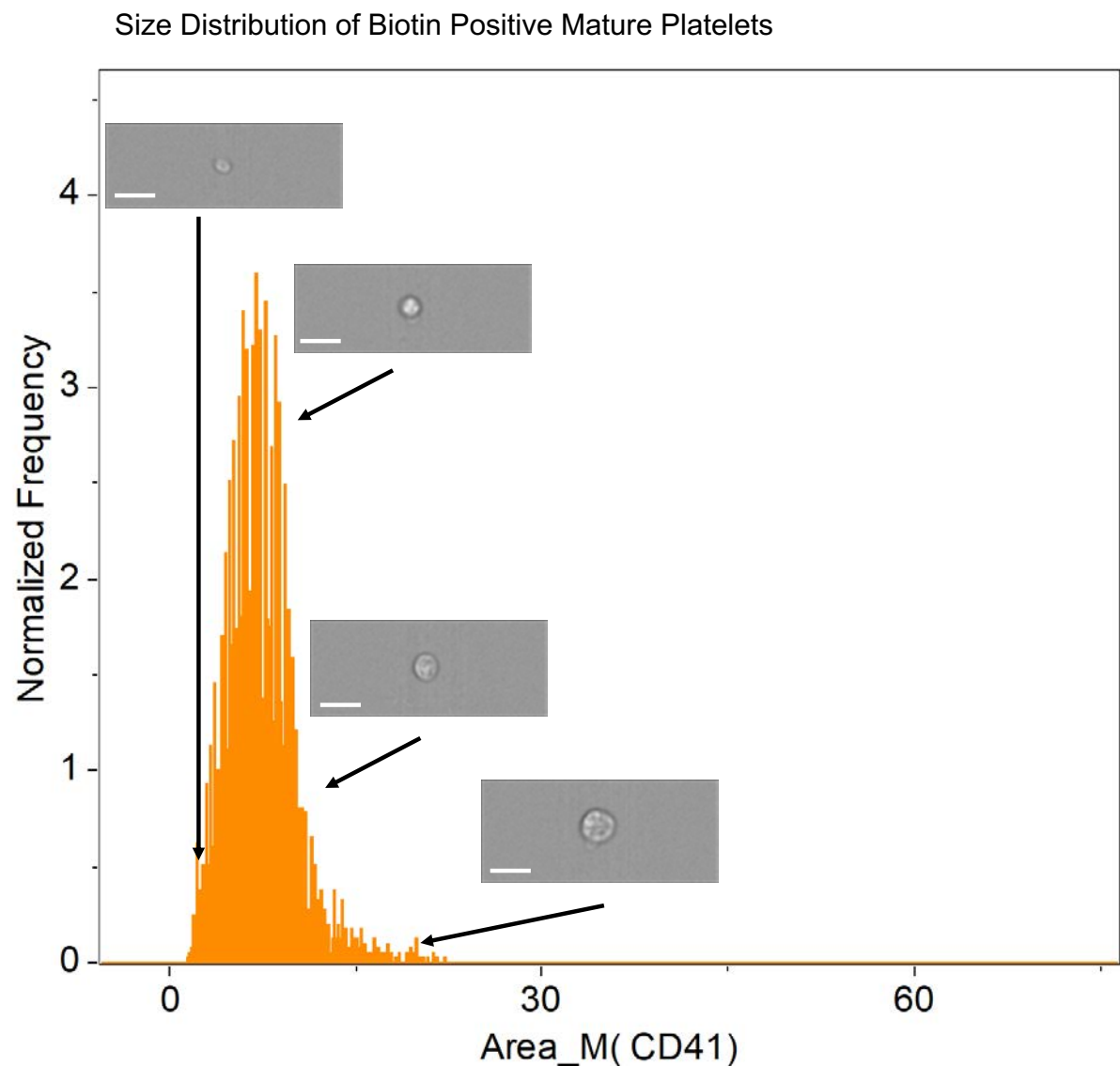


Figure 6.13: Size Distribution of Biotin Positive Platelets

A histogram to show the size distribution (determined by area of CD41a fluorescence morphology (M)) of biotin positive (mature) platelets ($n=4$) was measured by ImageStream^X at day 1 (see Figure 6.9). Examples of APC CD41a positive platelet images (determined by Brightfield imaging) are also included to demonstrate their approximate positional value (determined by black arrows) along the Area_M(CD41) axis.

6.4 Discussion

6.4.1 Preplatelet Maturation Events in Mice

To confirm our human findings, we initially showed in mice, preplatelets and their maturation events were not only preserved in 37°C citrate blood but also sphered when exposed to EDTA (Figure 6.1). Furthermore, SiR tubulin direct live cell labelling in citrate blood demonstrated the classical microtubule structure of murine preplatelet maturation events was analogous to humans (Figure 4.11 & 4.12 and see chapter 4 discussion for further description regarding marginal band morphology during preplatelet maturation). In mice, preplatelets contained a normal yet large circular marginal band ($>3\mu\text{m}$ in diameter) and upon conversion into barbell platelets the marginal band was reorganised into elaborate continuous figure-of-eight structures (Figure 6.2c). Murine and Human preplatelets also exhibited mean diameters of $\sim 3.2\mu\text{m}$ and $\sim 3.4\mu\text{m}$ respectively (Figure 6.3a & 4.13a respectively). Comparison of murine and human preplatelet and barbell platelet perimeter measurements also demonstrated that circulating barbells originate from platelets $>3\mu\text{m}$ in diameter in both species (Figure 6.3b 4.13b). Furthermore, as the barbell perimeter was significantly greater than preplatelets in both species (Figure 6.3c & 4.13c) it is possible, larger preplatelets ($>4\mu\text{m}$ in diameter) exhibit a greater tendency to convert into barbell structures (see chapter 4 discussion).

Interestingly, the maximum perimeter of human and mouse barbell platelets differed somewhat. In humans the average maximum perimeter was $\sim 41\mu\text{m}$ whereas for mice it was much larger at $\sim 61\mu\text{m}$ (Figure 4.13b and Figure 6.3b). These findings are counter-intuitive as on average, mouse platelets are smaller than human platelets (1-2 and 2- $3\mu\text{m}$ in diameter respectively) [484]. This suggests newly released platelets

are larger in mice compared to humans. This could potentially be due to differences in MK ploidy between species. However, despite this, both the human and murine data supports the notion that barbell platelets originate from large, newly released immature platelets

Although preplatelet maturation events in mice seem analogous to human, a key difference was observed when quantifying the fraction of preplatelets and barbell platelets in blood. In mouse blood, the fraction of preplatelets was less than that observed in humans (1.9 and 3.3% respectively; Figure 6.4a & Figure 4.14a). In contrast, the fraction of barbells in mice was greater (1.7 and 0.5% respectively). Interestingly, these fractions equated the same across species (3.6 and 3.8% respectively). Nevertheless, this suggests, that the total fraction of preplatelet maturation events in human and mice although remarkably similar, there could be subtle differences in the kinetics and lifespan of these structures. In humans, the platelet lifespan is 8-10 days [219] whereas, in mice it is approximately half (4-5 days) [6, 485]. As a result, it is likely, preplatelet maturation is more rapid in mice compared to humans suggesting internal mechanisms such as a biological clock could play an important role in this process. A key candidate for regulating the platelet molecular clock is the pro survival protein Bcl-xL [226]. Mason and colleagues showed genetic ablation or pharmacological inhibition of Bcl-xL has proven to decrease platelet lifespan. Furthermore, from the same study, deletion of Bak (pro death protein) corrects this adverse phenotype. Thus, our data regarding human and mouse preplatelet kinetics and from chapter 5 showing preplatelets and barbell platelets contain increased expression of markers of immaturity (Figure 5.1 and 5.2), support

the hypothesis that preplatelet maturation is restricted to newly formed immature platelets.

Another key discrepancy between human and mouse data was the fraction of immature platelets was doubled in our mouse studies 3.4 and 7% respectively; Figure 4.14a and Figure 6.4a). As IPF was used to determine immature platelets in humans and TO RP in mice, such discrepancies can be explained by differences in methodologies, particularly regarding specificity of nucleic acid dyes, concentration and labelling duration. For example, IPF (human) is measured using an oxazine-based propriety dye and is performed rapidly (~90 seconds) by an automated haematology analyser (XN1000, Sysmex) [362-364] whereas the TO assay was performed by flow cytometry with labelling taking ~45 minutes [353, 486]. Discrepancies between these two methods have commonly been reported in the literature and at best have shown a moderate correlation (reviewed in [487]). Both dyes non-specifically label nucleotides however, the IPF overcomes this by standardising dye concentration and incubation time between samples and laboratories. Furthermore, anatomical differences in dye labelling have been reported. TO labels total nucleic acid content but also non-specifically labels δ -granules [346, 360]. IPF on the other hand, strongly labels platelet mitochondrial RNA [370, 455]. In addition to variation in methodologies, one cannot rule out differences in the rate of platelet turnover between species (mentioned above) impacting upon the % of immature platelets.

6.4.2 Preplatelet Maturation Events in Mouse Blood Represent the Rate of Platelet Production.

In agreement with our human data (Figure 4.14 and 5.3, 5.4, 5.7 & 5.8), the results in chapter 6 suggest, quantitation of murine preplatelet maturation events in blood may represent the rate of platelet production (determined by a correlation with RP%) under steady state (Figure 6.4b, c & d) and in response to acute severe thrombocytopenia (Figure 6.6c & 6.6d). A key limitation to our human ITP study was unknown variation in disease aetiology (see chapter 5 discussion). For example, ITP can be associated with low - high levels of platelet production [289]. Often the condition is also inflicted with suppressed platelet production due to interference of T-cells and/or autoantibodies [293, 297, 299, 477]. As a result, we observed in some ITP patients exacerbated or normal fractions of circulating barbell platelets (Figure 5.4b).

To overcome this limitation, we utilised a mouse model of acute thrombocytopenia where mice were injected IV with a GPIIb/IIIa polyclonal antibody to induce rapid Fc-independent clearance of platelets. This model rules out variations in disease aetiology and creates an environment of severely low platelet count without affecting platelet production and exposing the organism to persistent pathological stress. Others have shown mice contain a greater ratio of large, young platelets following antibody-induced thrombocytopenia [488]. So, one can assume, preplatelet production is enhanced and therefore the number of circulating barbell platelets also increases.

In response to the GPIIb/IIIa challenge, murine platelet counts rapidly decrease reaching the nadir 24h later (>98% reduction; Figure 6.5). Platelet counts recovered by an approximate quarter on day 5 and importantly at this time point MPV and RP% was significantly increased suggesting the circulating platelet population contained a high

percentage of large, newly formed immature platelets. In comparison to baseline measurements (prior to GPIIb/IIIa injection) the fraction of preplatelets doubled at day 5 and this was mirrored by a 3-fold average increase in barbell platelets (Figure 6.6a & 6.6b). Furthermore, these fractions observed at baseline and day 5 positively correlated with the RP% (Figure 6.6c & 6.6d) suggesting circulating preplatelet maturation events are dependent on changes in platelet counts and therefore, represent immature platelets reflecting the rate of platelet formation.

One other study has shown the fraction of barbell platelets in WT mice increases day 4 following RAMPS infusion [380]. However, this fraction only increased 1 to 1.75%, less than double whereas, our study we observed on average a 3-fold and in some mice as high as 5-fold (Figure 6.6b). As the other study was performed in PRP it is probable preplatelets and/or barbells could be pelleted during the PRP centrifugation process. Although no preplatelet measurements were conducted these could also have been lost during PRP preparation and one could predict this fraction also increased as in our study (Figure 6.6a).

In humans, platelet size is also dependent or related to MK ploidy [335]. For example, in ITP, MK classes with higher ploidy (32 and 64N) increase. In contrast, in AA patients, the mean and modal ploidy is reduced. A study by Corash (1987) demonstrated in response to acute antibody-induced thrombocytopenia, mice also show shifts in MK ploidy classes [489]. However, no obvious changes in MK ploidy were reported 12, 24 and 36h post platelet depletion. However, at 48h, researchers observed a modal shift in MK ploidy from 16 to 32N and a significant increase in the 64N class. This shift in ploidy was rapid and short lived as after 120h (5 days), the MK ploidy returned to normal. This may explain why in our study, the RP%, MPV and the size of barbell

platelets observed at day 5 were significantly greater than those observed at day 0. Interestingly, mild thrombocytopenia $40 \times 10^9/L$ does not exhibit a shift in ploidy but did increase platelet MPV [489].

6.4.3 Barbell Platelets Originated from Newly Formed Platelets

Currently there are no specific immunological markers that can completely discriminate preplatelets from other platelets. However, they can be measured by size based on previous mathematical modelling [384] ($>3\mu m$ in diameter; see chapter 4 discussion for details regarding mathematical modelling). In chapter 5, it was established, following chemotherapy-induced BM ablation, preplatelets and IPF remained normal even in the absence of platelet production (Figure 5.8). Thus, if preplatelets are immature platelets and maturation occurs early in the platelet lifespan this suggests, not all platelets $>3\mu m$ in diameter are preplatelets and therefore, exhibit the capacity to mature into barbells. To explore this further, we utilised an *in vivo* biotinylation assay, in combination with ImageStream^x to determine the size and morphology of newly formed immature platelets compared to mature. Others have previously used *in vivo* biotinylation to determine the lifespan of platelets in mice (see above) [219, 485]. From these studies it was concluded platelets circulate for 4-5 days producing ~25% new platelets a day. In our *in vivo* biotinylation assay, we only observed ~10% new platelets 24h after biotin injections (Figure 6.8 & 6.9). However, our study was conducted in C57/6 mice whereas, the above studies were performed in C3H mice. Variations in MK ploidy distributions have been reported between these two species with C3H containing a modal of 32N whereas, C57/6 16N [490].

In vivo biotinylation has also been previously used to confirm RP are newly formed platelets with a comparatively short lifespan of 1-1.8 days before they mature and live for 4-5 days in total [5, 356]. Data in this thesis also demonstrated for the first time that barbell platelets derive from newly formed immature platelets as these structures imaged by ImageStream^X were all significantly negative for biotin labelling at day 1 post biotinylation (Figure 6.10). In contrast, preplatelets consisted of variable biotinylation (including negative and highly positive) matching that observed in mature platelets (<3µm in diameter). This confirms not all large platelets (>3µm in diameter) are immature and therefore exhibit the ability to mature into barbells (Figure 6.10).

In support of this, using ImageStream^X, we interrogated the size distribution of total biotin negative (immature) and positive (mature) platelets. From this analysis, it was clear both mature and immature platelets contained platelets ranging from small to large with barbells only present in the biotin negative platelet population (Figure 6.12 & 6.13). As a result, immature platelets were significantly larger than mature platelets (Figure 6.11). Furthermore, if blood were anticoagulated in EDTA, one can assume barbells would have been spheroid and no size difference would have been observed. Importantly, it cannot be disregarded, many small biotin negative platelets on day 1 may have already undergone maturation in lung microcapillary beds [200, 201] or post pulmonary vessels.

In the literature there are contrasting reports regarding platelet size and age [318, 327, 343, 488]. As discussed above and in chapter 5, in response to enhanced peripheral destruction of platelets through autoantibody-mediated processes, newly formed platelets increase in size [334, 491]. This is also the case for severe arterial diseases associated with increased consumption [341, 342, 492] and recovery following

chemotherapy-induced BM ablation [346]. Thus, in response to physiological stress, platelet production is regulated by other mechanisms such as IL-1 α -mediated MK rupture [338] or CCL5-mediated MK proplatelet enhancement [464] (discussed in chapter 5).

Although platelet size is dependent on age in the above conditions, under steady state production, no such correlations are found. One study in baboons showed, following incorporation of radioactive labelled methionine into MK, researchers discovered platelets released from these MK varied in size. There is also evidence to suggest platelet size and age do not necessarily correlate in normal human blood. Transfusion of radioactive labelled autologous platelets demonstrated platelets with higher density were cleared more rapidly than low density platelets suggesting immature platelets are less dense than old. Importantly, the size distribution of low- and high-density platelets did not differ.

6.5 Limitations and Future Work

A key limitation of this chapter was the use of mouse models to interpret and support our findings from our human studies. Although it has been shown the genome of these species is virtually the same with out of ~4000 genes studied <10 are present in one species and not the other, some differences have been demonstrated between platelets such as circulating concentration and MPV [493, 494]. Also, ultrastructurally, mouse platelets contain greater granule heterogeneity and finally, differences in the redistribution of MK IMS preceding platelet formation suggesting platelet release could differ slightly [484].

The method of blood taking also varied across our assays (such as in GPIb α platelet depletion), depending on the measurements taken. For example, tail bleeds were used for measurements performed by haematology analysers whereas, terminal bleeds were required for ImageStream^X due the volume of blood required and minimal platelet activation. As a result, individual mice were needed for each time during the platelet depletion assay. This made it difficult to determine kinetics. Also, determining preplatelet barbell kinetics on day 8 following platelet depletion (platelet rebound) would have theoretically shown the preplatelet and barbell population return to normal mirroring that of the platelet count and RP.

Researchers have previously shown that RP are equivalent to newly formed platelets by using in vivo biotinylation [5, 356]. Flow cytometry confirmed our efficiency of labelling with 100% positivity of platelets post biotin injection (x 2). Furthermore, ~10% of platelets were negative at day 1 and ImageStream^X analysis at 24h confirmed the presence of barbells and preplatelets/large platelets that were biotin negative at 24h compared to the biotin positive populations of platelets and large platelets. It was very striking that only barbell platelets were exclusively biotin negative. Ideally, as biotinylation has not been used in conjunction with ImageStream^X previously, additional TO labelling would have confirmed biotin negative barbells originate from RP. Also, our findings were limited by the 24h biotinylation time point. As thousands of platelets are formed in a second (in mice), measurements at earlier time points would have assisted in elucidating whether immature platelets are released large and mature into small platelets rapidly within the circulation.

Despite the above, the use of ImageStream^X in combination with in vivo biotinylation or other available cell tracker labelling is a powerful assay which can aid in elucidating and characterising immature platelets. This will be discussed in detail in chapter 7.

6.6 Conclusions

In this chapter we have shown preplatelet maturation events occur in mice sharing key analogies such as morphometric attributes, cytoskeletal structure and combined circulatory fractions as observed in our human studies (see Chapter 4 and 5). However, differences in kinetics between species was observed. In response to acute yet severe thrombocytopenia, the fraction of preplatelets and barbell platelets increased and importantly under these and resting state conditions, these fractions positively correlated with the RP% suggesting, quantitation of platelet maturation events indirectly indicates the rate of platelet production in mice. Importantly, in vivo biotinylation demonstrated barbell platelets originated from newly formed platelets however, not all platelets >3µm in diameter formed barbells suggesting, preplatelets are a subpopulation of large platelets.

Chapter 7 - General Discussion

7.1 Overview of Results

The terminal maturation and metamorphosis of a large BM MK into thousands of mature circulating platelets is not only fascinating but is notoriously difficult to study both in vitro and in vivo. In the 1990's, the discovery of TPO not only rejuvenated MK research but enabled cultured MK to reliably undergo terminal maturation and platelet production in vitro. Although the consensus view was that BM MK release platelets in a terminally differentiated state [151, 152], there was good historical evidence that MK and/or proplatelets could also circulate in the bloodstream and release platelets in the pulmonary circulation [187]. Interestingly, the concept of circulating proplatelets was originally described by Radley [152]. However, due to advances in imaging techniques and the availability of recombinant TPO, terminally matured MK were shown to metamorphose into proplatelet structures that can then release platelets in vitro [156-163]. Furthermore, in vivo labelling of BM MK, demonstrated that they could also release large cytoplasmic structures or proplatelets into the sinusoid vessels suggesting that platelet maturation events continue within the bloodstream [163, 174, 380, 381] and the pulmonary circulation [187, 200]. More recent evidence now further supports the lung as a major site of platelet biogenesis [200]. Furthermore, platelets from stored platelet concentrates have been shown to transform into elaborate proplatelet-like barbell structures in vitro, which is accompanied by an increase in biomass and enhanced protein synthesis. Remarkably, the barbell platelets could also undergo fission into two platelets, a process which increased the platelet count under platelet storage conditions [382]. Not only were these newly formed platelet progeny ultrastructurally and functionally similar to normal blood platelets, but this was the first description of cell division by an anucleated cell. This observation ultimately led to the

discovery of a new terminal stage of platelet maturation which takes place in the bloodstream through a platelet intermediate or progenitor the “preplatelet” [380].

Blood anticoagulated in EDTA, spheres barbell platelets into preplatelets [384]. This probably explains why barbell structures are not measured in blood counters and not routinely observed within blood films. Furthermore, as barbells cannot be easily discriminated using CFC, it is necessary to develop advance high throughput image-based techniques such as ImageStream^X (an IFC approach) for their accurate quantification.

Although platelet fission was previously described for the first time [380, 382], it was still unclear whether all platelets or just a subpopulation (e.g. RP or immature platelets) were capable of undergoing division. We therefore hypothesized that immature platelets are equivalent to preplatelets and undergo barbell formation and fission to subsequently regulate physiological platelet counts. If this hypothesis is correct and preplatelets are analogous to immature platelets, this suggests, quantification of preplatelets and barbells in whole blood, could provide a more accurate measurement of immature platelets as an indirect measurement of the rate of thrombopoiesis resulting in the improvement of the classification and treatment of both acquired and inherited thrombocytopenia.

An important aspect of this thesis was to design and optimise a high throughput IFC approach (using ImageStream^X) to not only accurately quantify preplatelets and barbells in whole blood but to further study the dynamics and regulation of platelet fission. In chapter 3, ImageStream^X was used to interrogate thousands of platelet images and design methodologies to quantify preplatelets and barbell platelets using CD61 or CD41 fluorescence (Figure 3.8 & 3.10). As others had previously

characterised preplatelets as large circular platelets 3-10 μ m in diameter [384], they were distinguished using circulatory and diameter of fluorescence morphology. In contrast, as barbells consist of an elaborate and unique morphology [380, 382] (Figure 4.11 & 4.12), these structures were discriminated using multiple morphometric measurements based on elongatedness, 2-lobed symmetry of fluorescence morphology and narrowness of the cytoskeletal line of the barbell shaft (Figure 3.8). In addition to the classical barbell platelet structures, barbell-shaped microaggregates were also detectable and subsequently measured and characterised to determine whether they were related to true barbell platelets or simply artefacts caused by ex-vivo platelet activation. These structures were not only measurable (Figure 3.11) but importantly could be eliminated from interfering with the accurate barbell analysis (This was a key focus of chapter 4). The limitations of using ImageStream^x are discussed in chapter 3 whereas, this chapter will highlight possible improvements to methodologies and future investigations.

In Chapter 4, using a combination of ImageStream^x and blood film imaging, it was concluded the appropriate conditions for studying preplatelet maturation events in blood were at 37°C in tri-sodium citrate anticoagulated blood, in agreement with previous observations [17]. Furthermore, utilisation of ImageStream^x and advanced widefield-based imaging of platelet microtubules also clearly demonstrated that barbell structures were derived from a single platelet undergoing this transformation with a continuous figure-of-eight morphology ([380, 382] & Figure 4.11 & 4.12). In contrast, barbell-shaped microaggregates were composed of two individual platelets (Figure 4.5 - 4.7), were positive for CD62p (Figure 4.4) in contrast to true barbell platelets that

were negative (Figure 4.9). As a result, microaggregates could then be eliminated from future analyses using CD62p fluorescence (see method Figure 3.8 & 3.12).

Once artefacts were carefully removed from the analysis, morphometric analyses performed by ImageStream^x confirmed barbell platelets originated from preplatelets >3µm in diameter (Figure 4.13). However, it was still unclear if all large platelets were immature and therefore equivalent to preplatelets.

Others have measured preplatelets and barbells in human PRP and suggested they are equivalent to immature large platelets [384]. In agreement with the above study and the central hypotheses of this thesis, quantitation of preplatelets and barbell platelets in 37°C citrated blood demonstrated these fractions were not only comparable to IPF measurements but strongly suggested preplatelets are indeed analogous to immature RP and thus, represent an indirect indicator of the rate of thrombopoiesis (Figure 4.14). However, when investigating preplatelet/barbell kinetics 3h post phlebotomy, these fractions did not appear to significantly change over time suggesting under resting conditions preplatelet maturation may only occur in vivo and may be dependent on regulators and shear forces [176, 338, 464].

Finally, in this chapter, circulating barbells were also completely absent in citrated blood containing eptifibatide (10µM; Figure 4.15d). As normal $\alpha_{IIb}\beta_3$ signalling is essential for normal proplatelet formation [186] and mutations (predominantly modifying the salt bridge linking the intracytoplasmic region of the α_{IIb} and β_3 subunits) can cause macrothrombocytopenia [267, 269, 273, 274, 442, 443, 495] it was hypothesised that $\alpha_{IIb}\beta_3$ outside-in signalling could also play an important role in initiating preplatelet/barbell conversion.

In chapter 5, TO, mitotracker and HLA I labelling as well as interrogation of SSC light, confirmed barbells originate from preplatelets which display enhanced nucleic acid and granule content, HLA I expression and number of mitochondria (Figure 5.2). However, preplatelets all displayed similar increases in the above except for HLA I expression. Studies involving flow cytometry and electron microscopy show immature RP also contain increases in nucleic acid content [349, 353], number of mitochondria [372, 455] and granularity [372]. However, nucleic acid dyes and mitotracker probes are known to non-specifically bind nucleotides [346, 496] and therefore, cannot discriminate immature platelets from large platelets particularly as they contain more granules than small platelets [497]. Thus, these approaches assume, all large platelets are immature. However, under resting conditions and disorders associated with giant platelets, platelet size and age are often independent. As HLA I is a cell membrane glycoprotein it is likely to be a more specific measurement of immature platelets [373, 498]. Furthermore, in vivo biotinylation demonstrates, HLA I expression significantly decreases 4-fold within the first 24h of the platelet lifespan [373]. Considering this, mature large platelets probably contain less HLA I expression than immature preplatelets. As the preplatelet measurement described in this thesis (Figure 3.2) also includes large platelets, this may explain why the overall HLA I expression is significantly reduced when compared to barbells.

Importantly, the quantification of barbells was shown to be equivalent to immature RP and the rate of platelet production in thrombocytopenia (ITP) associated with enhanced peripheral destruction and turnover of platelets (Figure 5.1, 5.2 & 5.4). Furthermore, these structures were also virtually absent in patients at the nadir of the platelet count following chemotherapy-induced BM ablation (Figure 5.8d). As platelet size in ITP is

dependent on MK ploidy [335] and is therefore, known to be a good indicator of platelet turnover in hyper-destructive disorders [452] it was unsurprising preplatelets also positively correlated with the rate of thrombopoiesis in ITP. However, both the IPF and % preplatelets were unchanged at the nadir of platelet counts following chemotherapy (Figure 5.8b &c). This further indicated that preplatelet measurement also includes some large platelets which are not immature. Furthermore, the IPF method is also poor at discriminating BM failure patients from normal [421]. This also supports the consensus that under some conditions platelet size and age are independent [327, 343].

In chapter 5, we also determined the physiological importance of preplatelet maturation by demonstrating that many preplatelets were converted into barbells and underwent fission in culture to increase the platelet count (Figure 5.10) – a process which may have been continuously repeated until the platelet diameter reached a terminal threshold of $<3\mu\text{m}$ in diameter (Figure 5.10 & 11). This not only confirms that platelet fission can occur in vitro but is independent of shear forces. We also further ruled out the possibility of microaggregate formation by double labelling platelets and showing that the barbells and mature platelets that had undergone fission were all derived from single parent cells (Figure 5.9d). Furthermore, as the incubation assay was performed in a purified system this therefore suggests there may be an unknown inhibitor of preplatelet maturation in plasma or blood.

However, despite this, ~12.5% of the original preplatelet population still remained after 24h. This further supports the consensus that not all large platelets are immature preplatelets and therefore cannot undergo transformation into barbells and undergo fission. Preplatelet maturation was also highly dependent on cytoskeletal

reorganisation through microtubule and actin dynamics (Figure 5.15) which is driven by mitochondrial respiration (Figure 5.13b) and is possibly initiated by $\alpha_{IIb}\beta_3$ outside-in signalling (Figure 5.14a) supporting our previous findings (Figure 4.15d). Respiring mitochondria and increases in mitochondrial DNA replication in conjunction with increases in protein synthesis and cell mass have also been reported during barbell formation [382]. Enhanced protein synthesis likely ensures critical housekeeping proteins are maintained for normal platelet function following maturation.

Finally, in chapter 6, quantitation of preplatelets, barbells and their maturation events were performed in mice showing differences between human and mouse kinetics with mice containing more barbells and less preplatelets than human indicating more rapid turnover in mice (Figure 4.15a & 6.4a). Given the known differences in platelet counts ($150 - 400$ & $700 - 1000 \times 10^9/\text{mL}$) and lifespan $8 - 10$ & $4 - 5$ days respectively between these species, this is unsurprising [6, 219]. As in human ITP, the % of preplatelets and barbells significantly increased in response to antibody-induced platelet depletion (Figure 6.6a & b) and both fractions positively correlated with RP% under resting and thrombocytopenic conditions (Figure 6.6c & d). These findings were expected as both RP% and IPF% have been shown to be good discriminators of thrombocytopenia associated with enhanced peripheral destruction [353, 355, 499].

As it has been previously definitively demonstrated that RP are equivalent to young platelets [5, 356], in vivo biotinylation experiments in WT mice were performed to determine whether preplatelets and barbells were also immature (Figure 6.8 & 6.9). In addition, the mean size of biotin negative platelets was significantly larger than biotin positive suggesting immature platelets are larger than old (Figure 6.11b). However, interrogation of biotin negative and positive platelet images at 24h showed immature

and mature platelets consisted of both platelets and preplatelets. Importantly all barbells were also biotin negative at 24h, definitively demonstrating that these are immature platelets [187, 200]. In contrast, the preplatelets were ~50/50 biotin positive and negative. From this, it was concluded, large circulating platelets are both immature and old and only the immature fraction of these platelets exhibit the ability to transform into barbell platelets (which are all biotin negative at 24 hours; Figure 6.10, 6.12 & 6.13).

Considering all the findings in this thesis, an updated model of preplatelet maturation within the circulation is demonstrated in Figure 7.1 identifying limitations in current immature platelet markers.

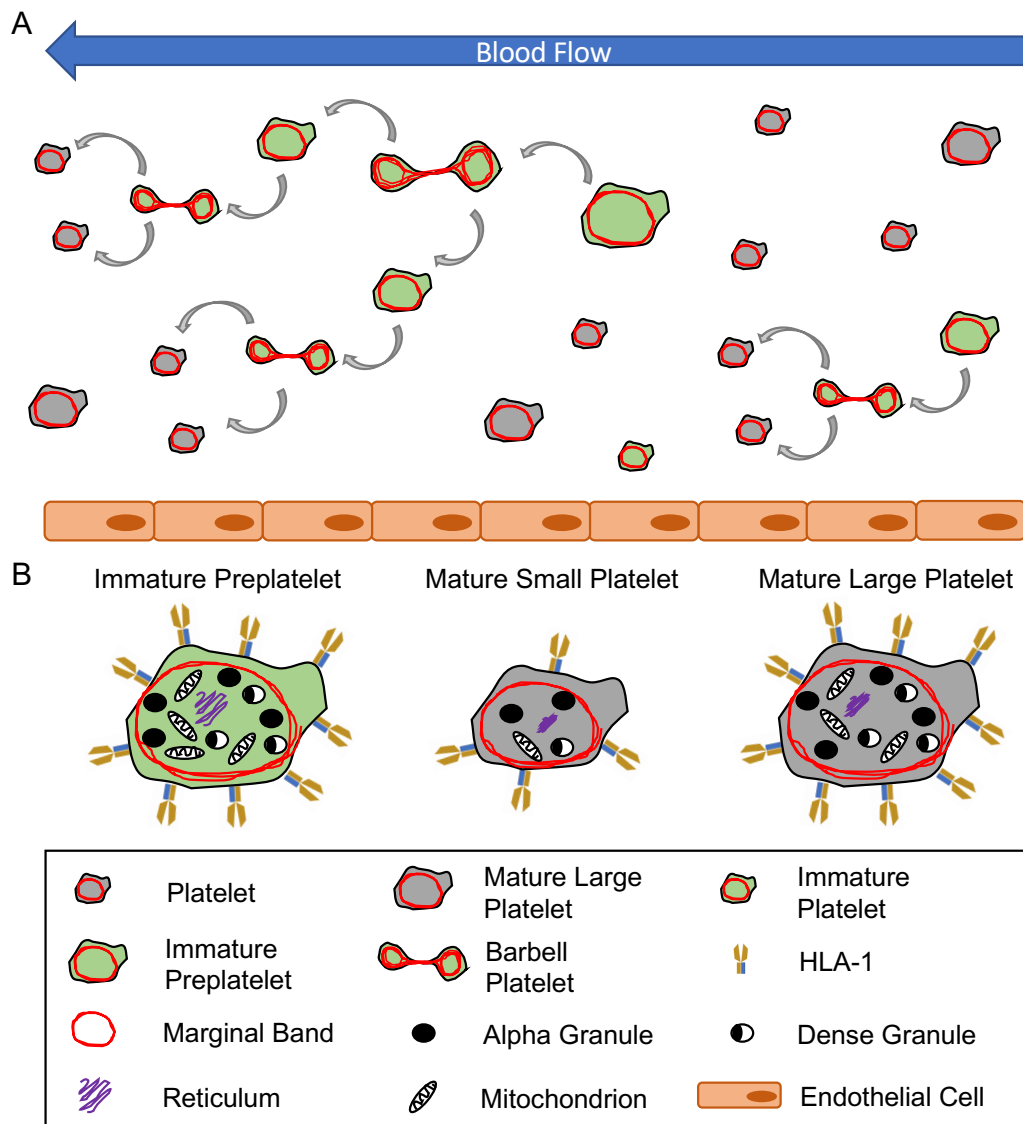


Figure 7.1: Model of Platelet Maturation in the Bloodstream

(A) Circulating platelets are heterogeneous in size and age. Immature platelets $>3\mu\text{m}$ in diameter are termed preplatelets (determined by a green cytoplasm). These platelet progenitor cells mature by continuously transforming into barbell platelets (possibly initiated by $\alpha_{\text{IIb}}\beta_3$ outside-in signalling) and undergoing fission into two smaller platelets until reaching a size threshold of $<3\mu\text{m}$ in diameter. Under steady state production, not all large platelets are immature. Unlike preplatelets, these lack the capacity to undergo maturation. Therefore, mature platelets consist of small and large platelets (determined by a grey cytoplasm). (B) Barbell platelets originate from immature preplatelets consisting of a greater nucleic acid content, granule content, number of mitochondria and HLA I expression compared to mature small platelets. In contrast, mature large platelets contain similar numbers of granules and mitochondria which are non-specifically labelled with dyes used for measuring IPF and RP. It is possible, large mature platelets may express slightly less HLA I than immature preplatelets which could potential separate preplatelets from mature large platelets. However, this needs to be determined.

7.2 Limitations of this Study

Using ImageStream^X, we quantified barbell platelets in both healthy human and mouse blood (~0.5% & 1.7% respectively; Figure 4.14 & 6.4). Using the barbell platelet % and estimate of platelet turnover, it is therefore possible to estimate the frequency of barbell platelet division required to maintain the normal platelet count to see if our estimates of barbell numbers are theoretically feasible. For example, in humans, 0.5% of circulating platelets are barbells at any given time (Figure 4.14). If we assume a total of 1 trillion (1×10^{12}) platelets are circulating in the human body containing 5L of blood, this will equate to 5×10^9 barbells. As human platelets survive for ~10 days, it is hypothesised the body replenishes 10% of the total platelet count a day, so 1×10^{11} platelets are required per day. If 5×10^9 barbells divide, they would produce 1×10^{10} mature platelets which means they would need to divide every 2.4 hours to maintain the count. In contrast, in mice 1.7% of platelets were barbells. If we assume a mouse has a total blood volume of 1.2mL and a platelet count of $1000 \times 10^6/\text{mL}$, they contain a total of 1200×10^6 platelets equating to 20.4×10^6 barbells. As murine platelets survive for ~5 days they must produce ~20% or 240×10^6 platelets a day. So, if 20.4×10^6 barbells divide, they would produce 40.8×10^6 mature platelets which mean they would need to divide every ~4.1h to maintain the platelet count. Although the human and mouse data are in the same order of magnitude and seem feasible, more detailed biotinylation or tracker dye labelling experiments with an increased frequency of blood sampling would help to confirm the lifespan of barbells in the blood before they undergo fission. Furthermore, these estimates not only assume that each barbell platelet produces 2 daughter cells the model does not consider maturation events which may have occurred within the lung.

A key limitation in this field and in this study is immature RP (including preplatelets and barbells) lack good, distinct markers for their resolution from mature platelets. In this study, and reported previously by others, size and platelet immaturity were independent [327, 343] suggesting immature or preplatelets are a subpopulation of large platelets. Despite this, preplatelets (determined as platelets $>3\mu\text{m}$ in diameter) still not only correlated with immature platelet measurements in human and mice (IPF% and RP% respectively; Figure 4.14b, 5.4c, 6.6c) but were characterised by significant increases in IPF, TO and mitotracker labelling and granularity – key markers associated with immature platelets. Many studies have shown platelet size (such as MPV) to be a good marker of thrombopoiesis [452] however, is often limited in disease context [347]. This was also observed in this study when measuring preplatelets in chemotherapy patients (Figure 4.8c). Furthermore, non-specific labelling of large platelets by TO and mitotracker probes [346, 496], which are known to contain more granules and mitochondria than small platelets [346] may explain such discrepancies in the data. As barbells express a significantly higher HLA I expression, this also suggests barbells originate from preplatelets with enhanced HLA I expression. Although, Angénieux and colleagues (2019) demonstrate cell surface expression of HLA I is a more useful marker of immature platelets [373], in this thesis HLA I expression does not seem to discriminate preplatelets from mature large platelets despite being significantly increased on barbell structures that are derived from this fraction. Data from this thesis and others has shown EDTA spheres barbell platelets (Figure 4.8a [384]). If immature preplatelets as well as their barbells express more HLA I than large platelets, a discrete population of HLA I positive platelets (observed by flow cytometry) should be more apparent in EDTA anticoagulated blood. Unfortunately this

measurement was not performed in this study or by Angénieux *et al* (2019) [373]. Furthermore, as it is suggested platelets have been shown to acquire soluble denatured HLA I protein from the circulation [375-378, 498], this highlights HLA I as a potential unsuitable marker of immature platelets. Thus, the data from this thesis and findings above emphasise the importance of using platelet morphology rather than just biomarkers for determining platelet heterogeneity and age.

As there is no definitive reference method for immature platelets, it is challenging to interpret new data in comparison to current IPF and RP measurements. TO labelling is highly non-specific (as mentioned above) and the RP% also increases with both TO concentration and incubation time [353]. Although some attempts have been made to standardise this method [361], interlaboratory results remain highly variable [500]. Furthermore, not all RP are large and platelet size is independent of age but dependent on MK development and therefore ploidy [343]. Although the IPF is standardised, this method still non-specifically labels the mitochondria of large platelets [421]. In this study the IPF% also did not significantly decrease from normal levels following chemotherapy-induced BM ablation. This agrees with previous studies that also show IPF not only lacks sensitivity for conditions of low platelet production or BM failure but can non-specifically label large platelets causing overestimation of immature platelets e.g., in macrothrombocytopaenia [421]. Furthermore, correlations between the IPF with RP% is at best moderate suggesting they require their own respective reference limits and values (reviewed in [365]).

If instruments such as ImageStream^X were to be implemented into a clinical setting, there are many challenges to consider such as expense (The cost of a basic 6 camera ImageStream^X is ~£500,000) and (if used as a standalone instrument within a scientific

laboratory) employment of specialised scientific staff for the operation and upkeep of the instrument. Although, preliminary data from this thesis demonstrates the percentage of circulating barbell platelets indirectly represents the rate of platelet production, additional studies (run within research funded laboratories) are required to determine the most appropriate disease type(s) prior to achieving diagnostic support. Following this, defining quality control measures would be essential to ensure best practice, common errors, good intra- and inter-laboratory clinical performance and therefore develop UK and international guidelines.

As the ImageStream^x requires laboratory bench space this imposes certain challenges regarding point-of-care and therefore rapid turnaround of treatment/results. For example, if the barbell measurement could be used to improve decision making when administering platelet transfusions, challenges concerning point-of-care would need to be resolved to meet rapid acute needs. Considering this, reach out to major haematology corporations would be essential to determine how measuring barbells would be incorporated into commercially available haematological instrumentation (discussed further below in section “7.3 Future Work”).

Many experiments in this study were generally performed in anticoagulated ex vivo blood samples and in vitro incubation assays. The field of ex vivo platelet production has demonstrated the importance of mimicking natural environments to accurately achieve and study platelet production [501-503]. The observations in this thesis were made in the absence of important environmental factors such as shear and turbulent force and ECM [176, 201]. These two factors have been shown to be crucial for normal platelet production. Furthermore, Thon and colleagues (2010) [380] showed isolated platelet intermediates consisting of preplatelets and proplatelets transfused into mice

rapidly undergo fragmentation or fission in the bloodstream to increase the platelet count. The rate in which this occurs is also much greater than that observed by culture and incubation assays used in this thesis.

To definitively show the efficacy of quantifying circulating barbell platelets in thrombocytopenia, much larger and wider clinical studies are required. Furthermore, as preplatelet/barbell conversion is inhibited in the presence of an $\alpha_{IIb}\beta_3$ inhibitor, it is possible preplatelet maturation may be inhibited in macrothrombocytopenic conditions related to defects in $\alpha_{IIb}\beta_3$ and other genes (see chapter 5 “Discussion”). However, unfortunately, no inherited congenital thrombocytopenic patient samples were available for this study.

Preplatelet maturation may also be inhibited in other conditions associated with giant platelets. Previous work by Thon and colleagues (2012) shows, preplatelet/barbell conversion is dependent on microtubule thickness and diameter [384]. Furthermore, giant platelet disorders such as MYH9-RD (May-Hegglin and Epstein’s syndrome) consist of platelets with up to a 20-fold increase in the number of peripheral microtubule coils [504]. Furthermore, immunofluorescence shows giant platelets consist of microtubules assembled into “balls of yarn” and failure to form a circumferential band results in spherocytosis. Thus, it is highly likely preplatelet maturation is inhibited in some MYH9-RDs. Also, conditions such as BSS (GPIb-IX mutations), are characterised by thrombocytopenia yet the majority of patients have normal numbers of MK residing in the BM (reviewed by [385]). Albeit, these MK form 50% less proplatelets with significantly larger nascent platelets compared to normal [505], it is still plausible inhibition of preplatelet maturation also contributes to the terminal size of these platelets.

7.3 Future Work

A recent publication has demonstrated, using ImageStream^X, it is possible to determine the DNA content and mitotic phases of Jurkat cells by extracting features based entirely on BF and darkfield (or SSC) imaging [506]. Such methods are developed through machine-learning algorithms either generated from fluorescent staining or by visually inspecting and categorising cell classes of interest. Machine learning has the ability to exclude unnecessary blood preparations such as incubations and fluorescent labelling therefore, generating machine learning algorithms for barbell platelet quantification means blood samples can be immediately fixed in a preheated fixative and citrate anticoagulant.

In addition to machine learning, in vivo platelet labelling antibodies DyLight X488 (green) and X649 (far red) by Emfret Analytics, now offer a new approach to directly label circulating platelets without inferring with their lifespan. For example, it is possible to label all circulating platelets with X488 and e.g. 24h later labelling with X649. Thus, all newly formed platelets (immature) are labelled red whereas mature platelets are green. Using these antibodies, researchers are now beginning to characterise and track immature platelets in mice and have suggested platelet size and immaturity are unrelated therefore, further supporting the notion not all large platelets are immature [507]. Utilising the above method, mice can be bled directly into a heated fixative permitting immediate analysis of blood. Using ImageStream^X, platelet morphology can be interrogated based on X488 and X649 fluorescence profiles. This assay was attempted in mice near the end of this project in collaboration with Queen Mary's in London. Unfortunately, the experiment was unsuccessful (due to poor labelling

efficacy). Due to the impact of the COVID19 pandemic, the experiment could not be repeated.

The use of in vivo platelet labelling antibodies therefore has great potential to aid in our future understanding of the heterogeneity of newly formed platelets through methodologies such as FACS sorting, the use of Image-based instrumentation (in this study) as well as genomics, transcriptomics, proteomics and metabolomics. As mentioned earlier in this thesis, preplatelets as well as the entire immature RP population lack specific antigenic markers for their complete discrimination from mature platelets. By utilising X488 and X649 labelling and FACS sorting, it would be possible to compare the entire proteomic profile of immature and mature platelets and assist in the discovery of new markers which can be incorporated into current haematology analysers for rapid clinical diagnostics.

The work from this thesis outlines the clinical potential of incorporating high throughput image-based methodologies into current haematology analysers to improve quantification of immature platelets. One potential candidate is the Cobas m511 (Roche, Basel, Switzerland). The Cobas m511 is a digital morphology analyser that rapidly generates complete blood cell classification and counts using Bloodhound® technology [508]. From 30µL of blood, a monolayer is printed onto a slide, where cells such as platelets are labelled and imaged. However, this method is essentially an automated blood film approach and may not be sensitive enough to provide an accurate quantification of barbell platelets within citrated blood samples.

7.4 Conclusions

Recently we have begun to successfully unravel the final stages of platelet formation which occur in the bloodstream. Using such critical information, we demonstrate preplatelet or immature platelet maturation occurs in a subpopulation of large platelets and the rate in which this takes place signifies the rate of platelet production and BM activity in health and disease, providing a platform to develop a new and improved approach to perhaps overcome the limitations of current immature platelet measurements. We propose quantification of preplatelet derived barbells could be an invaluable tool for diagnosing and managing thrombocytopenia. With the use of appropriate temperature regulated anticoagulants, fixatives and labels with incorporation of image-based analyses into future haematology analysers, this may offer a unique and more affordable automated approach than IFC to measure preplatelet-derived barbell structures in whole blood.

Chapter 8 - List of References

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Chapter 9 - Appendix

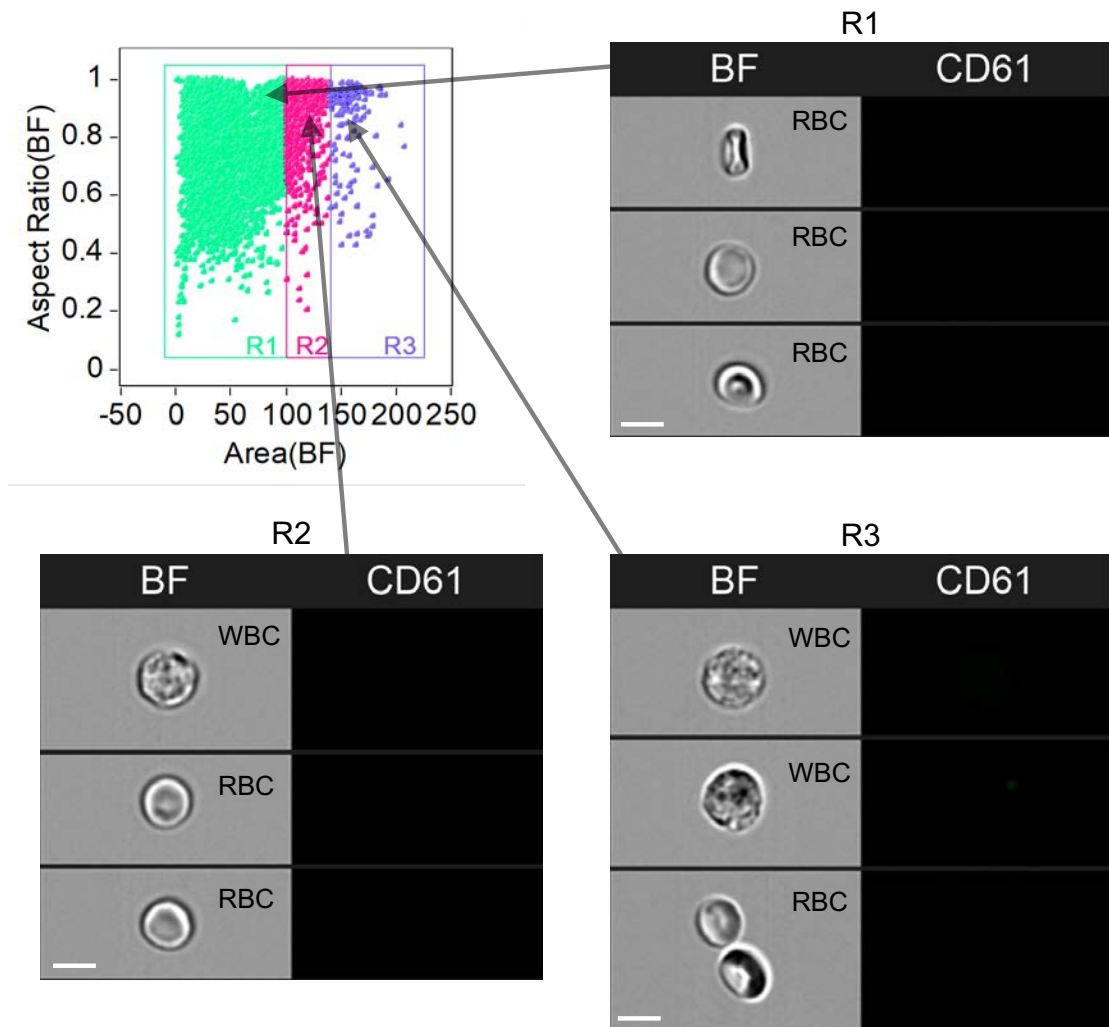
9.1 Appendix to Chapter 2

Appendix Table 2.1: Example of a Compensation Matrix Generated by the ImageStream^x

	CH01	CH02	CH03	CH04	CH05	CH06
CH01	1	0.118	0.118	0.044	0	0.001
CH02	0.105	1	1	0.044	0	0.001
CH03	0.012	0.132	0.132	0.194	0	0.002
CH04	0.005	0.055	0.055	1	0	0.001
CH05	0.003	0.018	0.018	0.473	1	0.073
CH06	0.027	0.027	0.027	0.095	0	1

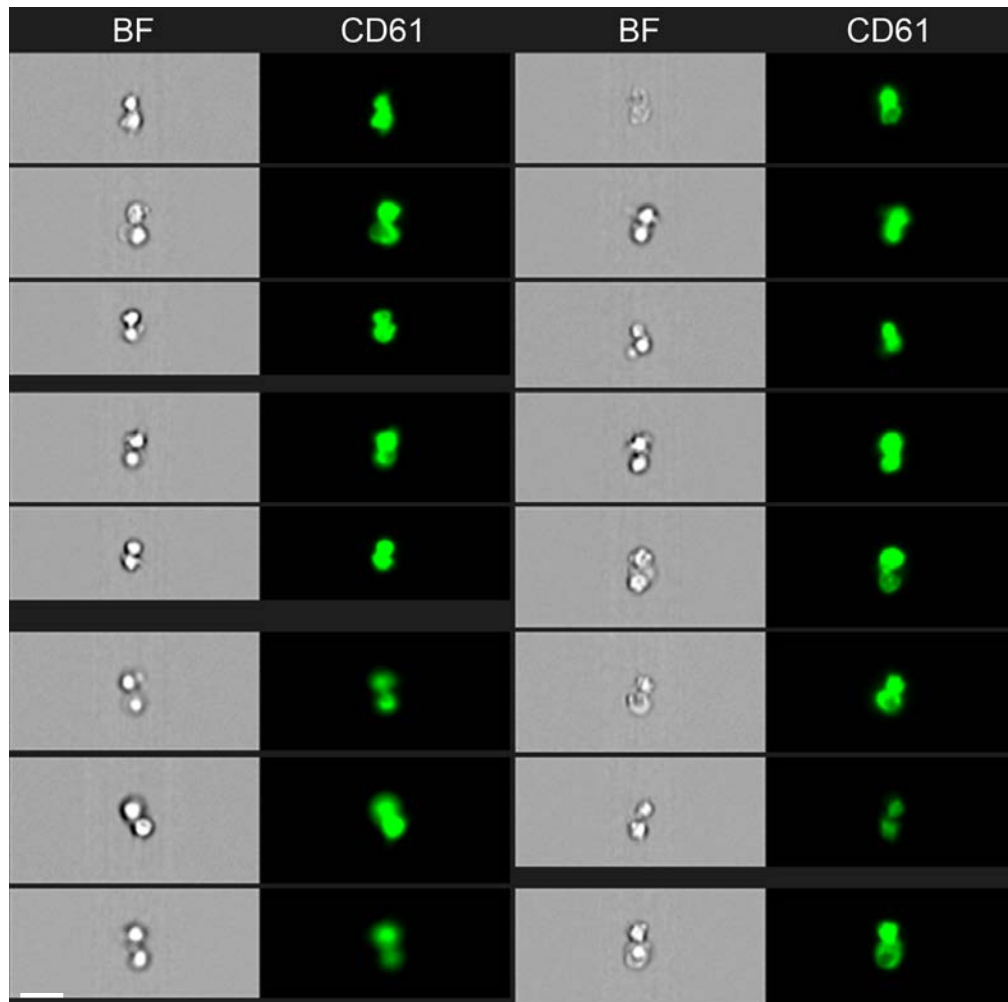
CH01: BV421 (CD62p); CH02: FITC (CD61); CH03: PE CD42b; CH04: Alexa Fluor 591 Mitotracker CMXRos; CH05 N/A; CH06 Brightfield.

9.2 Appendix to Chapter 3



Appendix Figure 3.1: Location of RBC and WBC within the Whole Blood Cloud

Healthy human citrated blood was labelled with FITC CD61 and 50,000 brightfield (BF) images were acquired by the ImageStream^x (x60 lens). All whole blood cellular images were plotted using aspect ratio and area of BF imaging. 3 regions were manually generated from the total cloud: R1 (mint green), R2 (magenta) & R3 (violet). Non-platelet cellular events were present in these regions (determined by CD61 fluorescence). Red blood cells (RBC) were situated at the far-right side of R1, throughout R2 and dual RBC events in R3. White blood cells (WBC) were predominantly present throughout in R2 & R3. Images are depicted by BF and CD61 fluorescence. Scale bar 7 μ m. Representative of a single experiment.



Appendix Figure 3.2: Barbell-Shaped Microaggregates in Tri-sodium Citrate Blood

Healthy human citrate blood was labelled with FITC CD61 and PE CD42b and 10,000 double positive images were acquired using the ImageStreamX (x60 lens). Barbell-shaped microaggregates were manually selected from the 10,000 platelet events and images were depicted using brightfield (BF) and CD61 (green) fluorescence. Scale bar 7mm. This figure is representative of a single experiment.

9.3 Appendix to Chapter 4

Appendix Table 4.1: Healthy Control (n = 52) Blood Count Data Generated by XN1000 Haematology Analyser (Sysmex)

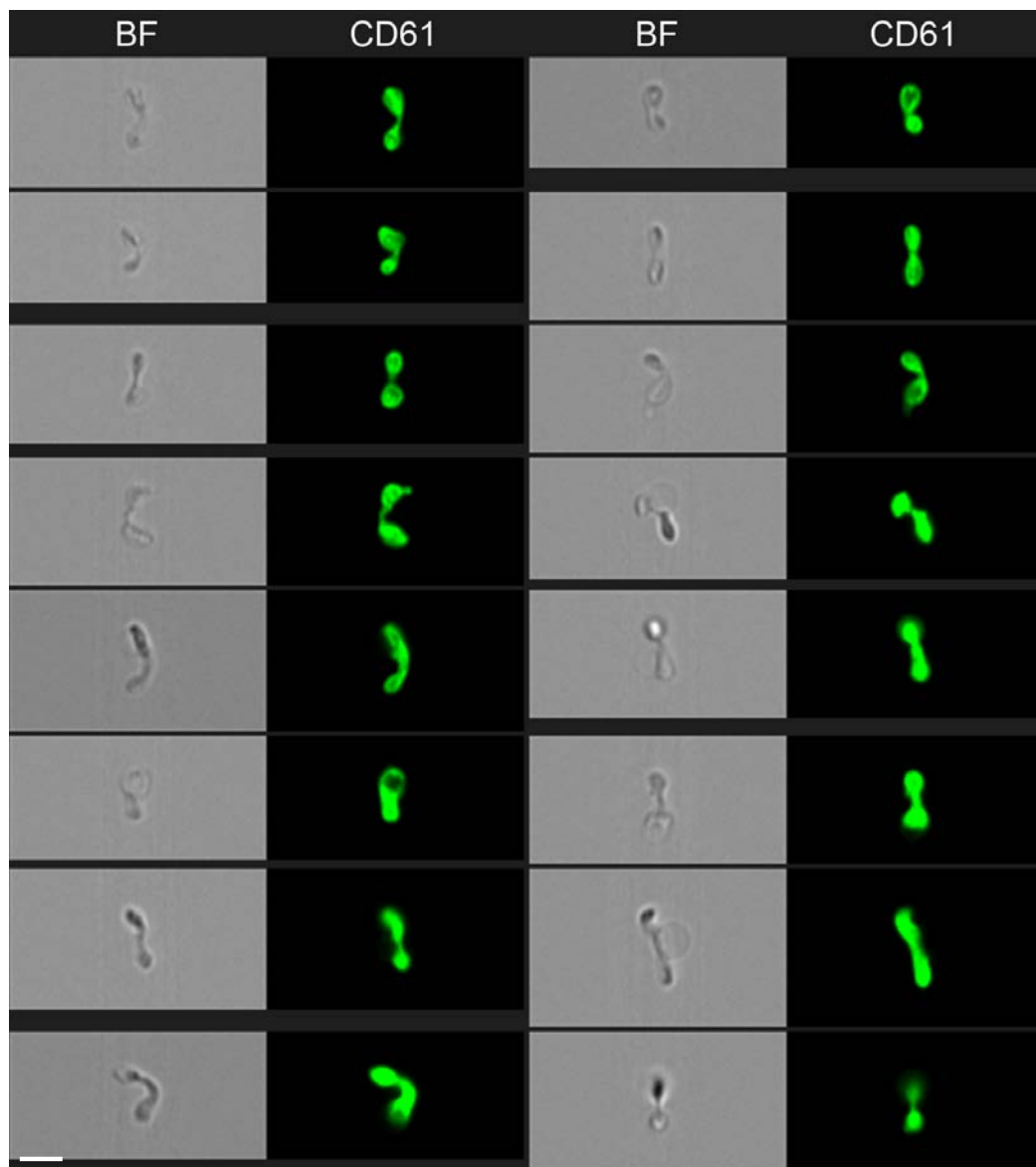
	PLT-F [10^9 / L]	IPF (%)	MPV
MEAN	251	4.14	9.70
RANGE	174 – 316	1.1 – 8.2	9.20 – 11.08

PLT-F: Platelet Fluorescence; IPF: Immature Platelet Fraction; MPV: Mean Platelet Volume.



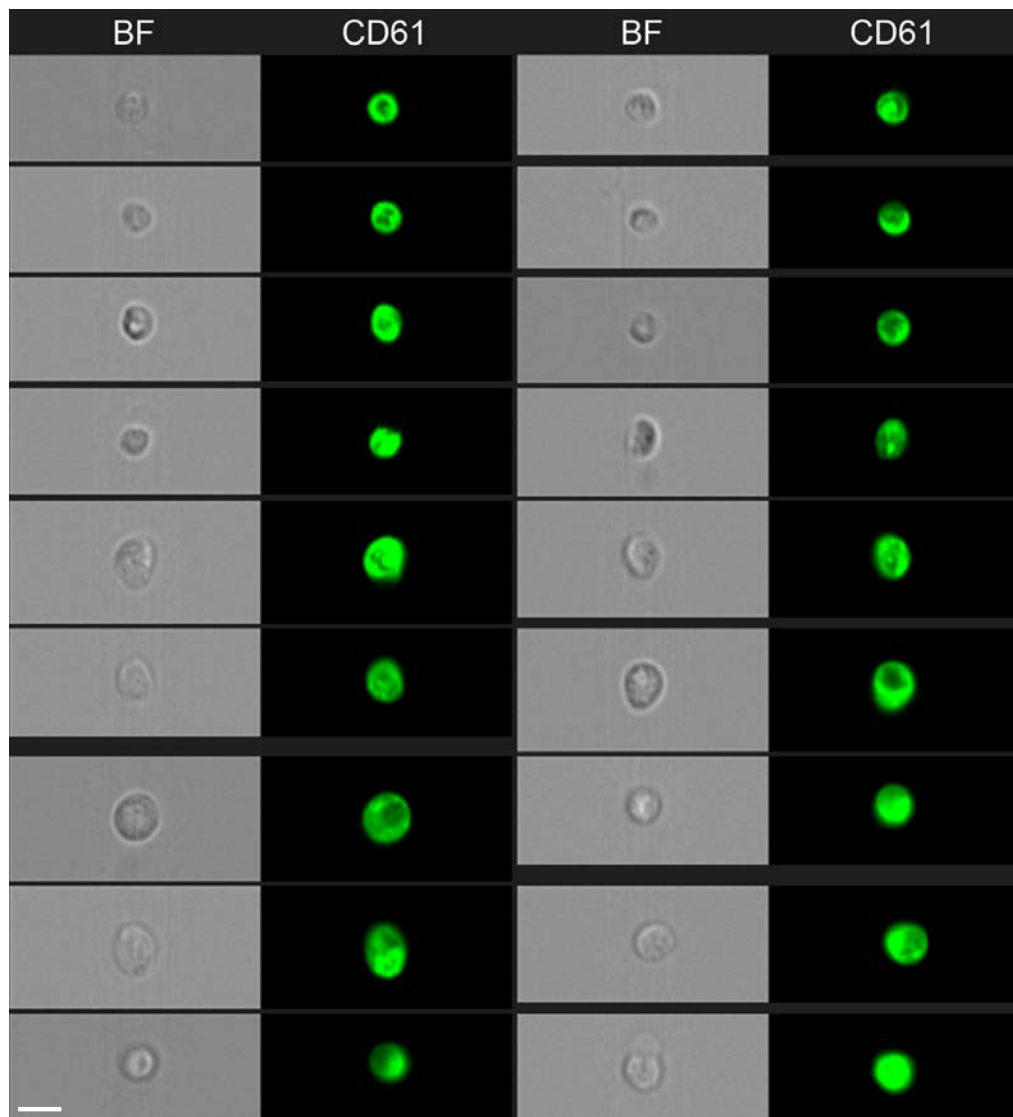
Appendix Figure 4.1: CD62p Localisation on Barbell-Shaped Microaggregates in Citrate Whole Blood

Healthy control citrate blood was labelled with BV421 CD62p, FITC CD61 and PE CD42b and 10,000 CD61/CD42b positive images were acquired using the ImageStream^x (x60 lens). Barbell-shaped microaggregates were manually selected from the microaggregate gate (Figure 3.11) and images were depicted to show CD62p localisation using brightfield (BF), CD61 (green), CD62p (red) and merged CD61/CD62p fluorescence. Scale bar 7 μ m. This figure is representative of a single experiment. Images are representative of a single sample.



Appendix Figure 4.2: Images of Barbell Platelets in 37°C Citrate Whole Blood by ImageStream^x

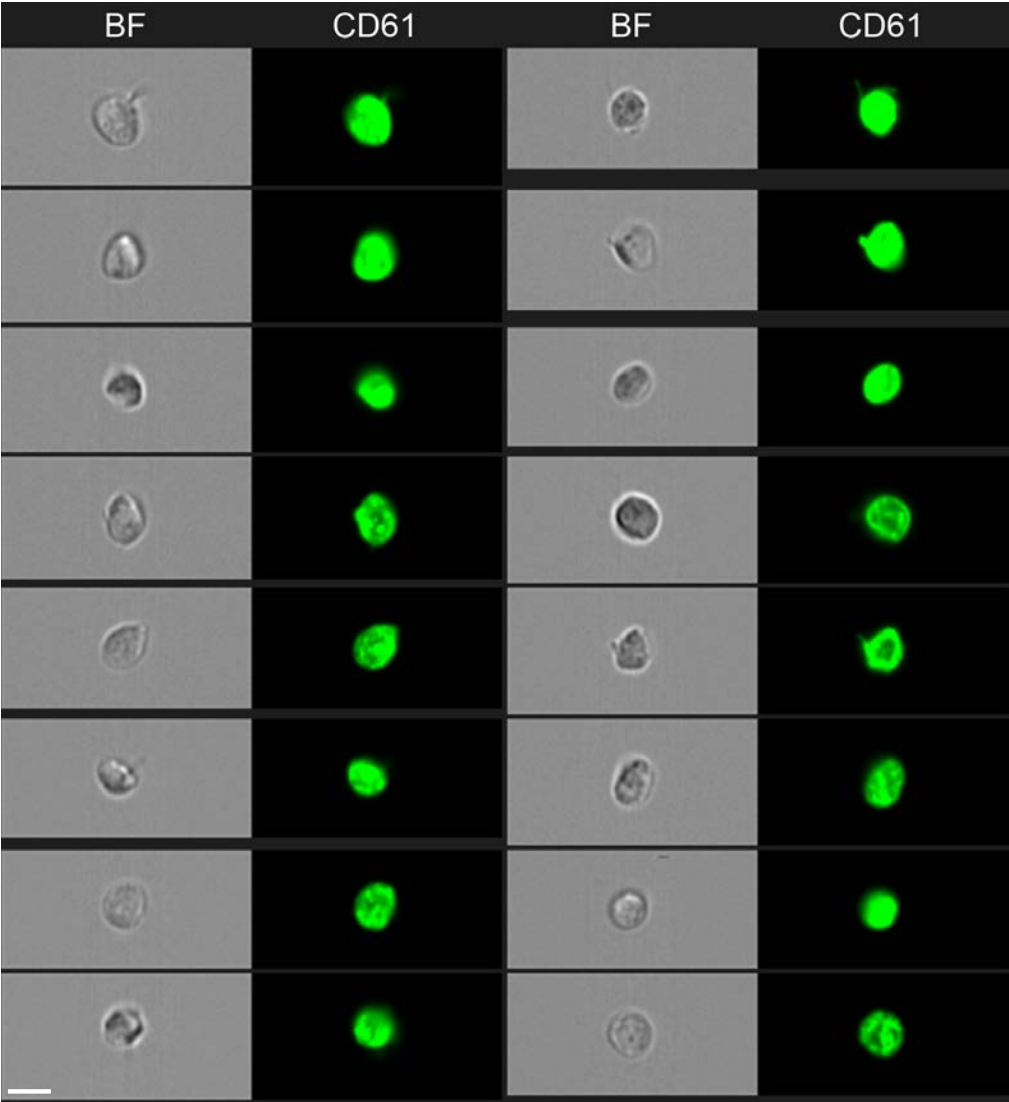
Healthy control 37°C citrate blood was labelled with BV421 CD62p, FITC CD61 and PE CD42b. 10,000 FITC CD61/PE CD42b positive platelet images were acquired using ImageStream^x (x60 lens). Barbell platelets were manually selected from the barbell gate (Figure 3.8) and images represented by brightfield (BF) and CD61 (green) fluorescence. Scale bar 7µm. Images are representative of a single sample.



Appendix Figure 4.3: Images of Preplatelets in 37°C Citrate Whole Blood by ImageStream^x

Healthy control 37°C citrate blood was labelled with BV421 CD62p, FITC CD61 and PE CD42b. 10,000 FITC CD61/PE CD42b positive platelet images were acquired using ImageStream^x (x60 lens). Preplatelets were manually selected from the preplatelet gate (Figure 3.8) and images represented by brightfield (BF) and CD61 (green) fluorescence. Scale bar 7µm. Images are representative of a single sample.

9.4 Appendix to Chapter 5

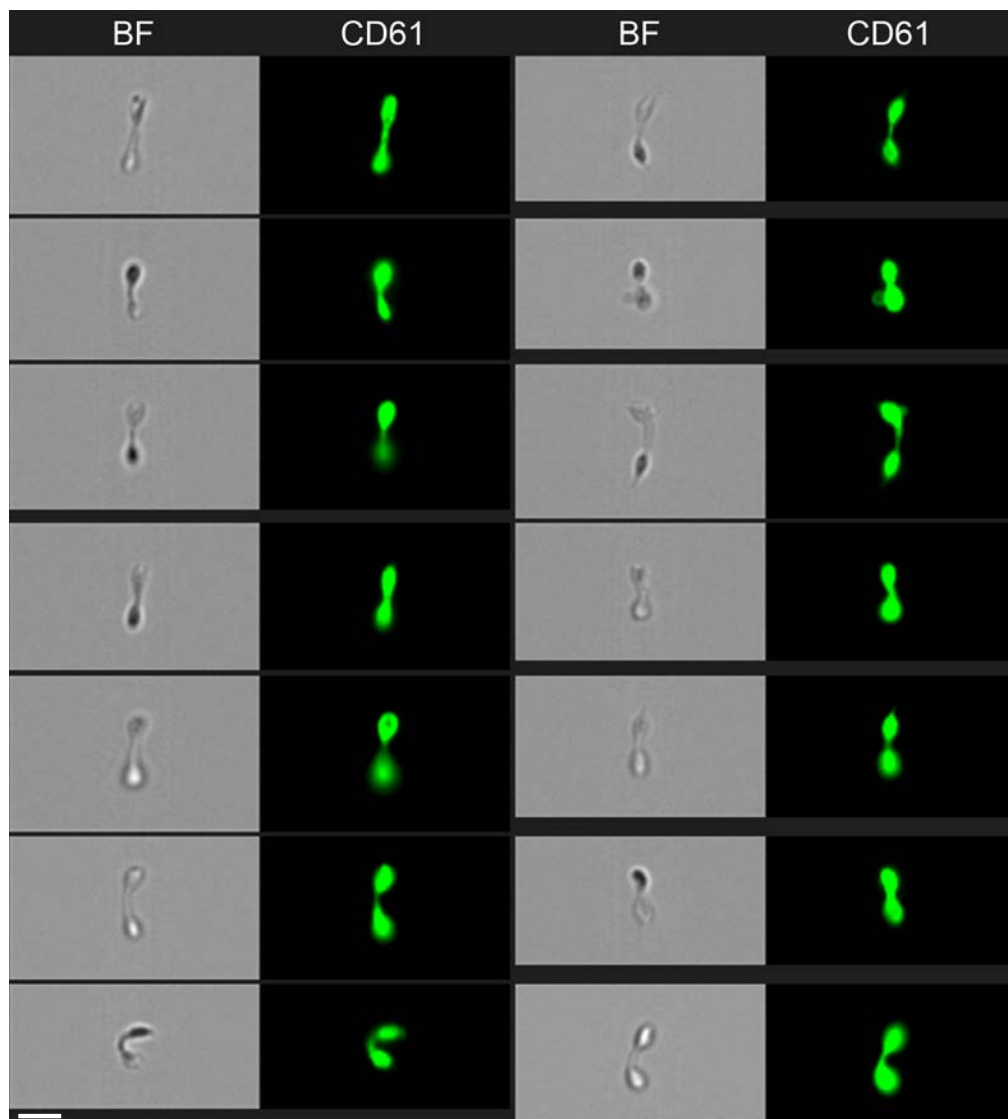


Appendix Figure 5.1: Images of Preplatelets in ITP Citrate Whole Blood
Immune thrombocytopenia (ITP) 37°C citrate blood was labelled with BV421 CD62p, FITC CD61 and PE CD42b. 10,000 FITC CD61/PE CD42b positive platelet images were acquired using ImageStream^X (x60 lens). Preplatelets were manually selected from the preplatelet gate (Figure 3.8) and images represented by brightfield (BF) and CD61 (green) fluorescence. Scale bar 7µm. Images are representative of a single sample.



Appendix Figure 5.2: Images of Barbell Platelets in ITP Citrate Whole Blood

Immune thrombocytopenia (ITP) 37°C citrate blood was labelled with BV421 CD62p, FITC CD61 and PE CD42b. 10,000 FITC CD61/PE CD42b positive platelet images were acquired using ImageStream^x (x60 lens). Barbell platelets were manually selected from the barbell gate (Figure 3.8) and images represented by brightfield (BF) and CD61 (green) fluorescence. Scale bar 7µm. Images are representative of a single sample.



Appendix Figure 5.3: Images of Washed Platelet Barbell Platelets Originating from healthy Control Citrate Blood

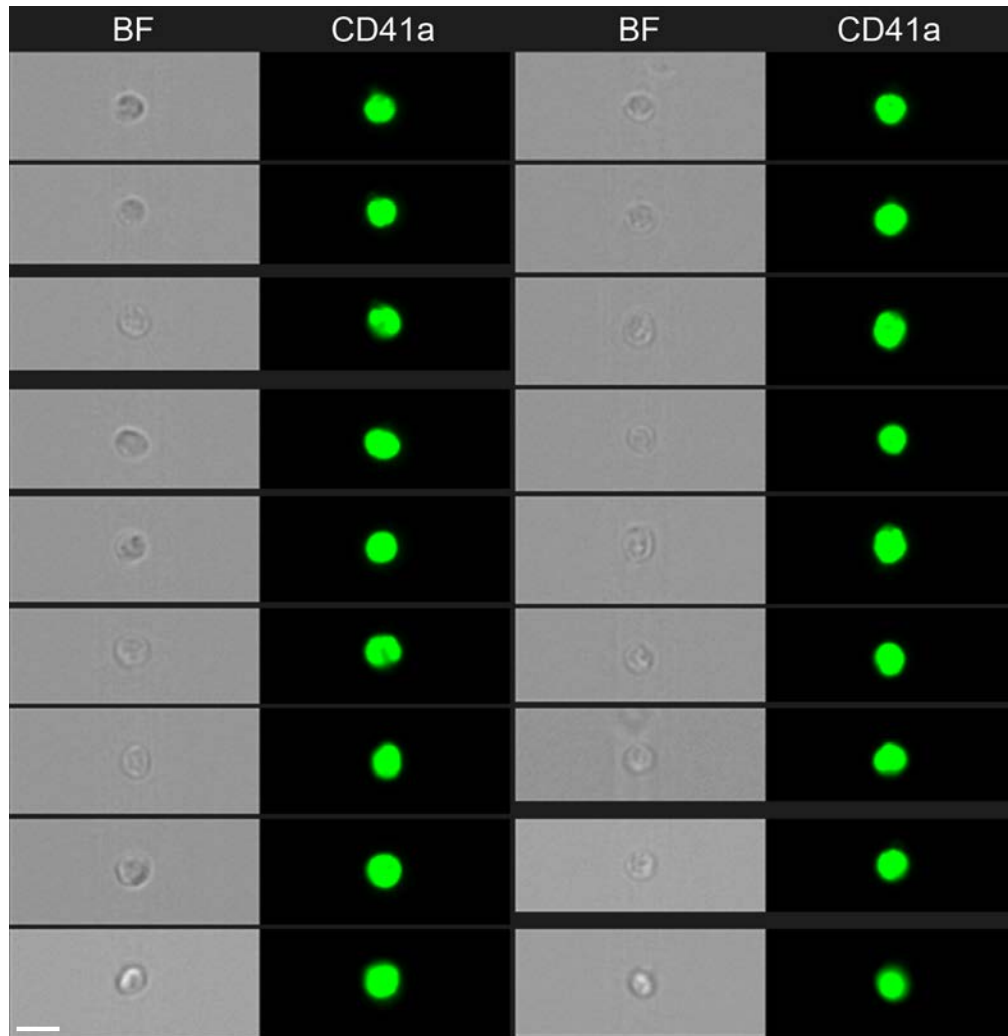
Barbells were imaged following a 6h incubation at 37°C. Washed platelets ($\times 10^6$ / mL) were labelled with BV421 CD62p, FITC CD61 and PE CD42b. 10,000 FITC CD61/PE CD42b positive platelet images were acquired using ImageStream^x (x60 lens). Barbell platelets were manually selected from the barbell gate (Figure 3.8) and images represented by brightfield (BF) and CD61 (green) fluorescence. Scale bar 7 μ m. Images are representative of a single experiment.



Appendix Figure 5.4: SiR Tubulin Labelling of Washed Platelet Barbell Platelets

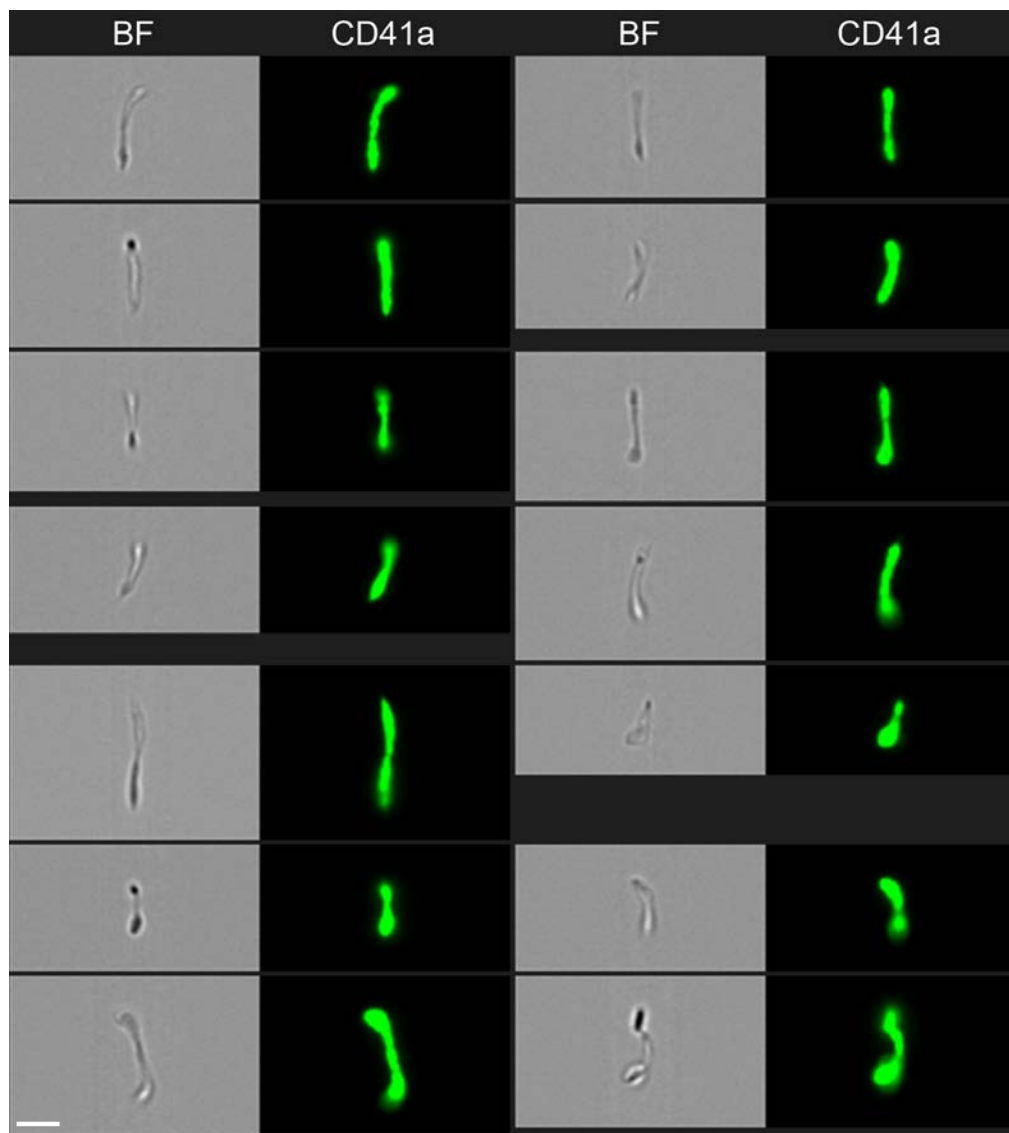
Barbells were imaged following a 6h incubation at 37°C. Washed platelets ($\times 10^6/\text{mL}$) were labelled with BV421 CD62p, FITC CD61, PE CD42b and AF674 SiR tubulin. 10,000 FITC CD61/PE CD42b positive platelet images were acquired using ImageStream^x (x60 lens). Barbell platelets were manually selected from the barbell gate (Figure 3.8) and images represented by brightfield (BF), CD61 (green) and SiR tubulin (Red) fluorescence. Scale bar 7 μm . Images are representative of a single experiment.

9.5 Appendix to Chapter 6



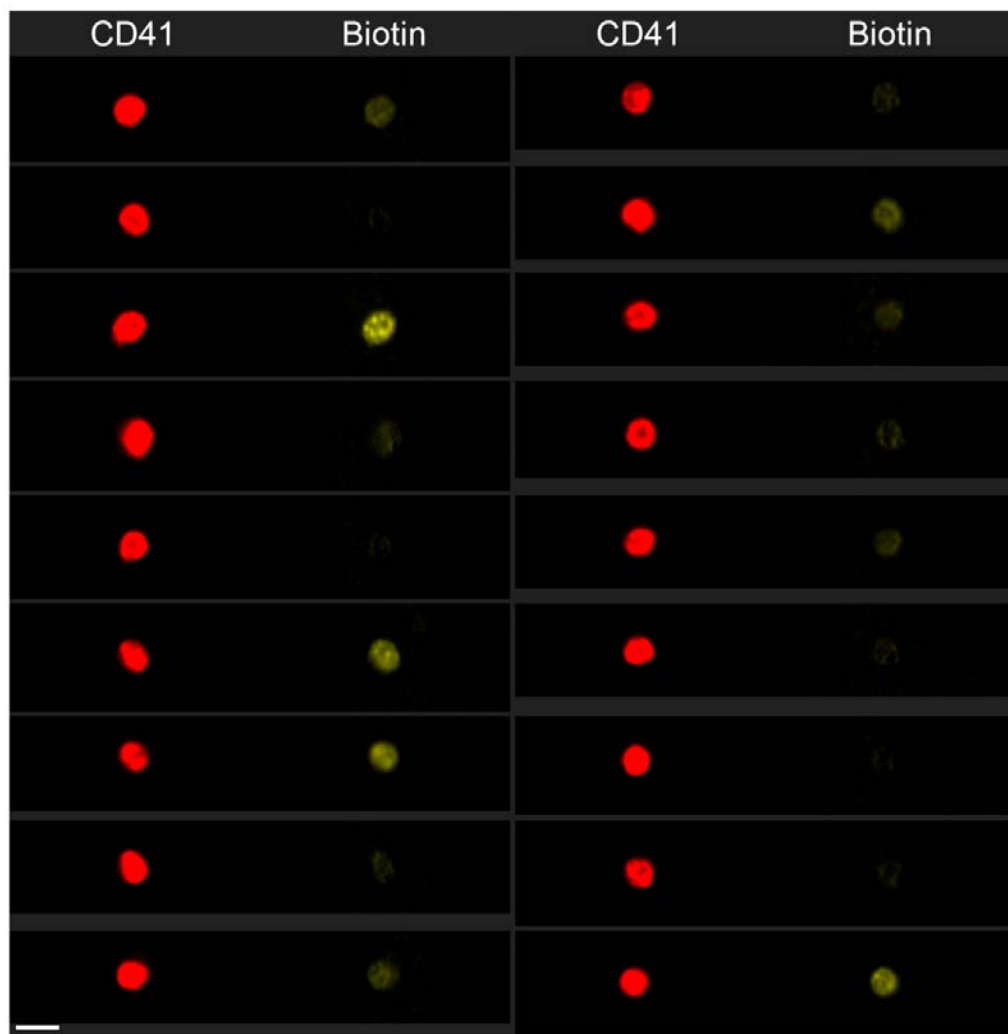
Appendix Figure 6.1: Images of Preplatelets in 37°C Murine Citrate Blood

Murine citrate blood was incubated for 1.5h at 37°C and labelled with BV421 CD62p and FITC CD41a. 10,000 CD41a positive platelet images were acquired using the ImageStream^X (x60 lens). Preplatelets were discriminated as in Figure 3.10 and manually selected. Preplatelet images are depicted by brightfield (BF) and CD41a (green) fluorescence. Scale bar 7µm. Images are representative of a single sample.



Appendix Figure 6.2: Images of Barbell Platelets in 37°C Murine Citrate Blood

Murine citrate blood was incubated for 1.5h at 37°C and labelled with BV421 CD62p and FITC CD41a. 10,000 CD41a positive platelet images were acquired using the ImageStream^x (x60 lens). Barbell platelets were discriminated as in Figure 3.10 and manually selected. Barbell platelet images are depicted by brightfield (BF) and CD41a (green) fluorescence. Scale bar 7mm. Images are representative of a single sample.



Appendix Figure 6.3: Preplatelet Biotinylation

Mice were injected intravenously twice with NHS-Biotin (4mg/mL; 30 min apart). On day 0 complete biotinylation was shown by flow cytometry (Figure 6.8a). 24h following the final biotin injection, mice were bled via terminal bleed into 4% citrate (1/10) and samples were incubated for 1.5h at 37°C and labelled with BV421 CD62p, FITC Streptavidin and APC CD41a. 10,000 CD41a positive platelets were acquired using ImageStream^X (x60 lens). Preplatelets were discriminated as in Figure 3.10 using APC CD41a fluorescence. Preplatelets were manually selected from the preplatelet population to show variation in biotinylation. Images are depicted using CD41a and biotin/Streptavidin (yellow). Scale bar 7µm. Images are representative of a single sample.



Appendix Figure 6.4: Barbell Platelet Biotinylation

Mice were injected intravenously twice with NHS-Biotin (4mg/mL; 30 min apart). On day 0 complete biotinylation was shown by flow cytometry (Figure 6.8a). 24h following the final biotin injection, mice were bled via terminal bleed into 4% citrate (1/10) and samples were incubated for 1.5h at 37°C and labelled with BV421 CD62p, FITC Streptavidin and APC CD41a. 10,000 CD41a positive platelets were acquired using ImageStreamX (x60 lens). Barbell platelets were discriminated as in Figure 3.10 using APC CD41a fluorescence. Barbell platelets were manually selected from the barbell population to show variation in biotinylation. Images are depicted using CD41a (red) and Biotin/Streptavidin (yellow). Scale bar 7mm. Images are representative of a single sample.