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Single Molecule Localisation and Structured Illumination Microscopy of Platelet Proteins

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"Running Head" – Super-resolution imaging of platelets

Abstract

Super-resolution microscopy has become increasingly widespread over the past 5 years and allows users to image biological processes below the diffraction limit of traditional fluorescence microscopy where resolution is restricted to approximately 250 nm. Super-resolution refers to a wide range of techniques which employ different approaches to circumvent the diffraction limit. Two of these approaches, Structured Illumination Microscopy (SIM) and Single Molecule Localisation Microscopy (SMLM), which provide a doubling and tenfold increase in resolution respectively, are dominating the field. This is partly because of the insights into biology they offer and partly because of their commercialisation by the main microscope manufacturers. This chapter will provide background to the two techniques, practical considerations for their use and protocols for their application to platelet biology.

Keywords

Platelets, Super-resolution, Single Molecule Localisation Microscopy, SMLM, Direct Stochastic Optical Reconstruction Microscopy, dSTORM, Structured Illumination Microscopy, SIM

1. Introduction

Platelets are small cells, densely packed with granules which contain a dynamic cytoskeleton that is key to their function. Historically, good quality imaging of platelets has been restricted to the use of Electron Microscopy (EM) which is able to give high resolution information of structures such as the cytoskeleton [1,2] and alpha and dense granules [3,4]. Indeed, EM has been used clinically in the diagnosis of certain granule related disorders such as Grey Platelet Syndrome (alpha granule) and Hermansky-Pudlak syndrome (dense granule) [5]. Whilst EM allows for high quality imaging of the ultrastructure of platelets, it is restricted to fixed and highly processed samples. Visualisation of specific proteins via immuno-gold EM is possible, but again it is technically more challenging than the traditional cell preparation for fluorescence microscopy. Fluorescence imaging offers many distinct advantages over EM, including the ability to easily label specific proteins of interest, perform multi-colour imaging and to follow dynamic processes in living cells [6].

Fluorescence microscopy has allowed for significant advances in our understanding of the microscopic structure of cells, and in particular the dynamics and relationships between multiple proteins or structures of interest. However a fundamental limitation of fluorescence for high resolution imaging is that, until recently, the resolution of the image was determined by the diffraction limit of light. First defined by Ernst Abbe in 1873, the diffraction limit of light was described as a physical restriction on the final resolution of an image as a consequence of spherical waveforms passing through a circular aperture [7]. Steadily improving optical configurations, including high numerical aperture objectives, which are able to capture more of the photons emitted from a sample, have brought the resolution limit down to ~250 nm in *xy* and ~500 nm in *z*. This means that any two points of light that are closer together than 250 nm in *xy* will be seen as one object instead of two when viewed using a light microscope [8]. Whilst widefield and confocal imaging have provided many unique insights into cellular structures and their localisation, a fundamental caveat has always existed when studying structures beneath this limit of spatial resolution. For example, when we consider that the size of vesicles and granules are in the region of 50 - 500 nm it becomes clear that co-localisation of two different proteins in a diffraction-limited image does not necessarily mean that the two proteins are found within the same compartment. These issues are compounded in platelets, which are small, densely packaged cells where spatial relationships more often lie beneath the diffraction limit. As such, diffraction limited imaging of platelets often leads to false co-localizations and overlap of fluorescent labels, making conclusions about their exact location and function more difficult to draw. Platelets also lack a nucleus, which makes them intractable to traditional cell biology approaches for fluorescently tagging proteins of interest.

Within the last 15 years sub-diffraction imaging, more commonly referred to as super-resolution microscopy, has been developed. Super-resolution microscopy is a term which encompasses a number of modalities, each of which has overcome the diffraction barrier using a different approach and therefore each one offers its own unique advantages and disadvantages. A comparison of the various techniques and their applications are beyond the scope of this chapter but various recent reviews [9,10] have described this in detail and are worth reading before embarking on any super-resolution

experiments. In this chapter we will introduce two super-resolution approaches: single molecule localisation microscopy (SMLM) and structured illumination microscopy (SIM), with a particular focus on their relevance to the study of platelets. We will provide protocols and hardware information on how to apply these techniques to the study of the platelet cytoskeleton and platelet signalling.

1.1 Single Molecule Localization Microscopy (SMLM)

SMLM methods allow for single molecule detection and the subsequent compilation of these localisations to form a super-resolved image [11]. SMLM techniques offer the highest spatial resolution currently available, down to as little as 10-20 nm in *xy*. This approach also offers unique quantitative benefits, particularly when investigating protein-protein interactions. Where single molecules are accurately detected and fitted, complex cluster analysis can be applied to study the density of individual emitters and their spatial relationship to one another [12,13]. SMLM techniques also pose a number of unique methodical challenges due to the sensitivity and nature of the detection method.

SMLM approaches first emerged as two principally similar but technically distinct imaging modalities. The first, STochastic Optical Reconstruction Microscopy (STORM), was published in 2006 [14] and relies on single molecule detections in fixed samples through the use of coupled dyes or antibodies. The second, PhotoActivated Localisation Microscopy (PALM), generates similar data sets through the use of unique photoactivatable or photoswitchable fluorescent proteins [15].

While a number of different STORM approaches have been developed, direct STORM (dSTORM) [16] is the most widely used due to its relative simplicity, the ability to utilise the optical sectioning capability of Total Internal Reflection Fluorescence (TIRF) and the option of performing 3D imaging by incorporating a cylindrical or astigmatic lens into the light path [17]. In dSTORM, a sample labelled with conventional antibodies is imaged at high laser intensities in the presence of an appropriate redox buffer. Under these conditions, the fluorophores are driven into a dark state, and only a small fraction of these become fluorescent in any one time frame. Therefore, a labelled sample is reduced into what is described as a ‘blinking’ data set, where a series of frames (in the region of

10,000-30,000) are acquired as the basis for computational reconstruction with each ‘blink’ representing a single fluorophore. Over the course of a large number of rapidly acquired frames, fitting algorithms can be applied to compile the probabilistic position of each emitter/molecule, thus rendering a super-resolved image.

While dSTORM can undoubtedly generate stunning images at 10s of nanometers of resolution, there are a number of technical requirements which must be met in order to achieve high quality data sets [11,18]. Firstly, an imaging system with a high NA (numerical aperture) objective, high powered lasers, and efficient camera is required. Modern EMCCD and CMOS cameras have evolved in leaps and bounds both in terms of speed and sensitivity, allowing for the acquisition of 100s of frames per second, thereby satisfying the need for the rapid acquisition of blinking fluorophores. In addition, the use of *z* drift correction systems such as Nikon’s Perfect Focus System (PFS) helps improve the quality of the final images displayed.

Beyond the necessary hardware, sample preparation and image reconstruction are two key components of successful SMLM imaging. With the appropriate camera, illumination, and acquisition parameters, a sequence of several thousand frames comprised of ‘blinks’ will be generated. This pointillistic data set is ultimately the source of a SMLM image, and as such appropriate computational modelling of these blinks is necessary for an accurate final super-resolved image. Early SMLM methods applied a relatively simple Gaussian fitting approach, whereby each individual point is fitted to a Gaussian curve, and the resulting compilation of fits assembled to generate the final image [11]. While effective, this approach suffered from a number of distinct limitations which has since led to the development of a host of alternative calculations for the derivation of SMLM images from blinking data sets. These are detailed in an excellent comparative review [19] and so it is advised that fitting algorithm selection be determined based on the characteristics of your sample. Regardless of the fitting method used, the number of photons detected from a single fluorophore is a major factor in determining the precise location of that molecule and experimental set ups should therefore try to maximise this parameter to achieve the image with the highest possible resolution [20].

Finally, sample preparation is another critical component of quality SMLM imaging. For dSTORM, where antibodies are applied to a fixed sample, generating a sample which attains a high labelling density and low background is essential. Firstly, an appropriate method of fixation and permeabilisation must be selected, particularly when imaging the cytoskeleton. For example, clearing with microtubule stabilizing buffer (MTSB) and subsequent methanol fixation can effectively remove non-polymerized tubulin molecules, thereby removing a significant amount of background from tubulin present in the samples. However, such a buffer may not be appropriate for imaging the fine actin filament network as this can be destroyed by methanol based fixation methods [21].

Quality antibodies are needed to maintain adequate labelling while minimising background. In dSTORM imaging individual points are assigned to the final reconstructed image, as such extracellular background can quickly clutter a reconstruction. This not only impacts the qualitative value of the image, but can have a significant effect on quantification. Labelling density is of equal importance at nanoscale resolutions, where poor sampling can significantly impact the final results [22].

Where the above criteria are satisfied, SMLM approaches, and in particular dSTORM, are powerful qualitative and quantitative tools with significant biological applications. Single molecule resolution allows for a robust interrogation of the spatial arrangement of individual proteins within the cell. Where receptor dynamics are concerned, molecule clustering can provide a unique insight into how proteins are recruited in response to particular stimuli [23-25]. Examples of how this can be applied to the study of platelets are provided below. Similarly this approach can provide an unprecedented understanding of cytoskeletal structures, all of which lie significantly below the diffraction limit. For example, actin exists in filaments 7-9 nm in diameter and microtubules are in the order of 25 nm, both of which play an integral role in platelet morphology and function. Therefore SMLM studies of the cytoskeleton and its relationship with key cellular components can reveal novel insights at the single molecule level [26].

1.2 Structured illumination microscopy (SIM)

In contrast to SMLM methods which detect single molecules and reconstructs the image in a pointillistic manner, structured illumination microscopy (SIM) is actually an optical sectioning technique which can increase axial resolution to around 100 – 120 nm [27,28]. In a fluorescence image, the sub-diffraction limit detail is known as high frequency information and is beyond the range of frequencies that can be collected by the objective lens. During SIM image acquisition, a low frequency grid pattern is projected onto the sample (usually via a diffraction grating in the illumination pathway) which generates interference patterns (known as Moiré fringes) due to interactions with the high frequency details of the sample. The interference patterns are of a lower frequency than the original detail and so can be collected by the objective lens and importantly these patterns contain information regarding the sub-diffraction limit fine sample detail. During imaging, numerous orientations of the grid pattern are taken at each focal plane (usually 5 phase shifts at 3 rotations) and the resulting interference patterns are mathematically reconstructed in Fourier space to extract sub-diffraction limit details of the sample. The technique can be performed in 2D and in 3D and furthermore has the advantage that it can be performed on live samples [29,30]. Assuming the appropriate lasers, diffraction gratings and filter sets are in place, SIM can image a range of standard fluorophores, including organic dyes (FITC, TRITC, Alexa, Atto, etc.) and fluorescent proteins (GFP, RFP, mCherry, etc.). As for SMLM imaging, high quality optics, cameras and lasers are required and the use of focal drift correction mechanisms will improve image quality. However, SIM is technically less challenging to perform than SMLM and is therefore more flexible in its application and can be more easily performed on a range of sample types (e.g. fixed and mounted, live and unmounted). As for all microscope techniques and discussed above, good fixation and labelling protocols are required, as is the need for good quality, validated antibodies against your protein of interest. The range of reconstruction algorithms for SIM is more limited than SMLM techniques, but Denmerle *et al* [27] describe important considerations when image processing and spotting common artefacts.

So, whilst SIM may not provide quantitative information on the 10 nm scale, excellent images can be generated due to the doubling of resolution, optical sectioning and increased contrast it provides. This can reveal details about the sample which are not clear in diffraction limited imaging. Furthermore,

because a SIM image is made up of pixels (and not coordinates as in SMLM), these can be treated as "standard" images and thus a full suite of commonly used image analysis techniques are available (e.g. Co-localisation by Pearsons Correlation) for image processing.

1.3. Super resolution imaging of platelets

Recently a number of platelet studies have employed super-resolution microscopy to investigate the spatial distribution of proteins within platelets to try to gain insight into their function. SIM is the technique which has been most widely used, most likely due to the ease of which it can be applied to samples that have been prepared for conventional fluorescence microscopy. Several groups have used SIM to investigate the co-localisation of proteins at higher resolution, for example the co-clustering of proteins into alpha-granules [31] or to see the spatial relationship between actin and adhesion related proteins such as vinculin [32,33]. In the case of Poulter *et al.* the increased resolution afforded by the use of SIM allowed the authors to develop a model for the spatial organisation and the role that a specialised actin structure, called the actin nodule, plays in platelet adhesion. This would not have been possible with conventional, diffraction limited microscopy [33].

SIM has also been proposed as an alternative to EM in the diagnosis of platelet granule disorders, with Hermansky-Pudlak syndrome as the proof-of-principle [34]. In this paper, the authors present SIM of CD63 in platelets combined with automated analysis of the images as a quantitative, unbiased, high-throughput method for diagnosis of this syndrome. Recently SIM has been used to image binding of GPVI-Fc to collagen fibres [35]. This study also used an alternative super-resolution technique known as STimulated Emission Depletion (STED) which is a confocal based technique offering resolutions in the range of 50 – 75 nm [36]. dSTORM has also been applied to the analysis of platelet receptors, where its quantitative nature has been exploited. For example, Poulter *et al.*, in addition to using SIM, applied dSTORM to identify patterns in the distribution of αIIbβ3 integrin at actin nodules and to quantify the level of phosphoprotein present at these structures [33]. Pollitt *et al.* used dSTORM to show that the podoplanin receptor CLEC-2 clustered above the level expected of a random distribution when platelets were spread on podoplanin. This work went on to show that it was

this clustering that was important for CLEC-2 signalling [37]. Other work looking at GPVI clustering in response to different substrates has shown that the density of GPVI clusters is determined by the substrate on which the platelet is spreading and that this clustering of GPVI dimers represents a second level of control to regulate GPVI-mediated signalling and platelet activation [37].

Whilst at present PALM has limited applications in the platelet field due to the need for genetically expressed fluorescent proteins, current work on CRISPR-Cas9 engineering of mouse lines and iPS cells, combined with developments in platelet production from these progenitor cells [38-40] may allow for the generation of platelets expressing photoswitchable fusion tags which, in turn, could permit quantitative super-resolution imaging of endogenous proteins, potentially in live platelets. This is an area that will undoubtedly flourish over the coming years.

2. Materials

2.1. Biological

Prepare all solutions using deionised water unless otherwise stated.

1. Concentrated citrate solution: 4 % (w/v) Sodium citrate

2. Acid Citrate Dextrose (ACD): 120 mM sodium citrate, 110 mM glucose and 80 mM citric acid

3. Modified Tyrode's buffer: 134 mM NaCl, 0.34 mM Na_2HPO_4, 2.9 mM KCl, 12 mM $NaHCO_3$, 20 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, pH 7.3.

4. Phosphate Buffered Saline (PBS): 10 mM phosphate buffer, 2.7 mM potassium chloride and 137 mM sodium chloride, pH 7.4

5. Prostacyclin: (0.1 µg mL^{-1}) in 50 mM Trizma base, pH 9.1

6. Biological substrate of interest. e.g. Fibrinogen (VWF and plasminogen depleted) diluted to 100 µg mL^{-1} in PBS

7. 35 mm glass-bottomed imaging dishes with the 10 mm diameter, no. 1.5 coverslip.

Comment [ST(oCaEM1]: Jon – we have a manuscript which has been revised and sent back to Sci Reports, that would go nicely here. If it is accepted and in time, is there a possibility we could include it here?

Absolutely – this could be added later – at the proof stage if this takes some time.

Comment [ST(oCaEM2]: Excellent – Thank you. Just need the reviewers and journal to pull their finger out and be nice to us!

Comment [JG3]: Could you please add a little more info here please as this is a little ambiguous…

Comment [ST(oCaEM4]: Detail added

Comment [JG5]: Could you please give the composition (even if from premade tablets, the composition should be known).

Comment [ST(oCaEM6]: Detail added

Comment [JG7]: Please state what the diluent is

Comment [ST(oCaEM8]: Diluent added

Comment [JG9]: If possible from alternative suppliers then the publishers don't like to include specifics for one supplier (they are trying avoid being sued!). If this does only work with dishes from this supplier then please reinstate the supplier.

Comment [ST(oCaEM10]: Other types of dishes can be used and so happy to have supplier removed here

8. 13mm diameter no. 1.5 coverslips

9. Fatty acid free BSA – denatured: 5mg mL^{-1} in PBS

10. Fixative: 10% formalin

11. Block buffer: 1 % BSA, 2 % goat serum in PBS

12. Phalloidin – AlexaFluor-488: 6.6 µM stock in methanol

13. Mouse anti-phosphotyrosine antibody (clone 4G10)

14. Goat anti-mouse IgG (H+L) highly cross absorbed AlexaFluor-647 antibody.

15. Non-fluorescing aqueous ~~Hydromount~~ mounting medium

16. STORM blinking buffer: PBS containing 100 mM 2-Mercaptoethylamine (MEA-HCl), 50 µg mL^{-1} glucose oxidase and 1 µg mL^{-1} catalase

2.2. Hardware

2.2.1 General hardware

Particle count and size analyser

2.2.2 SIM

SIM imaging is generally performed on commercial SIM microscopes, however, custom built systems do exist. Regardless of the source of the system, the following components are required for performing ~~single molecule localization~~structured illumination microscopy. Critical to the system is the ability to generate the illumination patterns onto the sample and this can be done using a variety of approaches which are detailed in Denmerle et al [27]. The system should be based around a research quality inverted fluorescence microscope stand with a high numerical aperture (NA), 100x oil immersion objective lens and excitation, dichroic and emission filter sets suitable for the fluorophores to be used. For performing 3D-SIM, a motorized stage is required to allow different focal planes to be

Comment [JG11]: Nf this type of mounting medium is the only that work please leave in – if other possibilities exist remove 'hydromount'

Comment [ST(oCaEM12]: Other mounting media can be used so happy to have tradename removed here. I have added a generic description.

Comment [JG13]: Spell out in full (and abbreviate if necessary)

Comment [ST(oCaEM14]: Detail added

Comment [JG15]: It might be best to focus on the specifications that are required and then go on with the following sections as examples of what you use. If you have access to the last book have a look at Chapter 1 section 2.3 where Marijke and Johan do exactly this type of thing. There is nothing wrong with suggesting what you use, but we need to be clear that there are alternatives

Comment [ST(oCaEM16]: I have tried to include a section for both SIM and STORM that lists the generic requirements of the system and then the specifics of what was used for the experiments as described. Please see what I have wrote below and let me know if this OK?

captured. Illumination of samples can be performed using standard lasers of the required wavelength with commonly used wavelengths including 647 nm, 561 nm and 488 nm and 405 nm. Both Scientific Complementary Metal-Oxide Semiconductor (sCMOS) and Electron Multiplying Charged Coupled Device (EMCCD) cameras can be used for SIM and the choice of camera will depend on the sensitivity or speed required. Finally, the system requires software to control both the acquisition of raw data and the processing of reconstructed data sets. Both freely available and commercial software packages are available to perform these tasks.

For the SIM experiments described below, we used a Nikon N-SIM system including an Eclipse Ti-E inverted microscope, Perfect Focus system 2, Apo TIRF 100x Oil DIC N2 1.49NA objective lens and 488 filter cube. Illumination was from a Nikon LU5 laser bed (including 488 nm laser line) and images were captured using an Andor DU-897 X-6005 EMCCD camera. The system was driven and images reconstructed using NIS-Elements Advanced Research software v4.5, with SIM module.

2.2.3 STORM

dSTORM imaging can be carried out on commercial STORM/PALM microscope or on custom built systems. Regardless of the source of the system, the following components are required for performing single molecule localization microscopy. The system should be based around a research quality inverted fluorescence microscope stand with a high numerical aperture (NA), 100x oil immersion objective lens and excitation, dichroic and emission filter sets suitable for the fluorophores to be used. Furthermore, focus drift mechanisms can improve the quality of the dSTORM data sets obtained by reducing drift in *z*. Illumination of samples should be performed using high power lasers of the required wavelength to drive fluorophores into the dark state. Commonly used wavelengths for dSTORM include 647 nm, 561 nm and 488 nm. A 405 nm laser is also required for reactivation of 647 nm dyes during imaging. Capture of single molecules requires a high sensitivity EMCCD camera with high readout speeds. However, recent advances in sCMOS cameras means they have started to be used on dSTORM microscopes. dSTORM can be performed either in TIRF or in 3D. This requires appropriate TIRF illuminator mechanisms or point spread function (PSF) distorting lenses in the

emission pathway to be installed to allow this additional functionality. The ability to tightly control the temperature of the microscope and thermal drift of the sample, along with the reduction in air movements across the sample will improve the stability of the system ~~of~~ for the timescale of the image acquisition and therefore improve the overall image quality. Finally, the system requires software to control both the acquisition of raw data and the processing of reconstructed data sets. Both freely available and commercial software packages are available to perform these tasks.

For the dSTORM experiments described below, we used a Nikon N-STORM system including an Eclipse Ti-E inverted microscope, TIRF module, Perfect Focus system 3, CFl SR Apochromat TIRF 100x Oil 1.49NA objective lens and N-STORM filter cube (Quad or single). Illumination was from an Agilent Ultra High Power Dual Output Laser bed equipped with 4 laser lines (30 mW 405 nm; 120 mW 488 nm; 120 mW 561 nm; 170-mW 647 nm) and images were captured using an Andor IXON Ultra 897 EMCCD camera. 3D images were captured using a 3D cylindrical lens and temperature was controlled by an Okolab S.r.l incubator set at 28 °C. The system was driven and images reconstructd using NIS-Elements Advanced Research software v4.5, with STORM module v4.1

3. Methods

3.1 Platelet preparation

The protocol detailed here gives information on how to isolate, wash and spread human and mouse platelets onto fibrinogen and then label and image the actin cytoskeleton (using fluorescent phalloidin) or phosphorylated proteins (using the 4G10 antibody). The antibody concentrations and labelling protocol for your proteins of interest will need to be empirically tested but this gives a good starting point for your experiments.

3.1.1 Preparation of washed human platelets

All steps are performed at room temperature and in a swing bucket rotor centrifuge unless otherwise stated.

1. Take human blood by venepuncture into sodium citrate (10% v:v). Further anti-coagulate blood by adding ACD to a final concentration of 10% v:v.

3. Spin anti-coagulated blood at 200 x g for 20 min. Carefully collect the platelet rich plasma (PRP) and centrifuge at 1,000 × *g* for 10 min in the presence of 0.1 µg mL^{-1} prostacyclin.

4. Resuspend the platelet pellet in 28 mL of wash buffer (25 mL of modified Tyrode's buffer, 3 mL of ACD (both warmed to 37 °C) and 0.1 µg mL^{-1} prostacyclin) and centrifuge the cells at 1,000 × *g* for 10 min. Resuspend the pellet in 1 mL of modified Tyrode's buffer.

5. Measure the concentration of platelets using a particle count and size analyzer and dilute to $2x10^8$ platelets mL^{-1} with modified Tyrode's buffer. Rest platelets for 30 min before starting the spreading experiments to allow excess prostacyclin to lose activity.

3.1.2 Preparation of washed mouse platelets

All steps are performed at room temperature and in a swing bucket rotor centrifuge unless otherwise stated.

1. Draw mouse blood into 10% v:v ACD by either direct cardiac puncture or from the vena cava following laparotomy. Add anti-coagulated blood to 200 µL warmed modified Tyrode's buffer.

2. Centrifuge anti-coagulated blood for 5 min at 2000 rpm in a benchtop microfuge. Decant the ~~top 750 µL of~~ PRP and a small amount of red blood cells (to ensure maximum recovery of platelets) into a clean 1.5 mL Eppendorf tube and centrifuge at 200 × *g* for 6 min. Carefully collect the plasma and the layer of platelets sitting on top of the packed red blood cells ~~layer~~ and place into a clean 1.5 mL Eppendorf tube.

3. Centrifuge the PRP (the plasma and platelet layers from step 2) at 1,000 × *g* for 6 min in the presence of 0.1 µg mL^{-1} prostacyclin in order to isolate the platelets from the PRP.

4. Resuspend the platelet pellet in approximately 200 µL modified Tyrode's buffer and measure the platelet concentration using a cell counter. Dilute the platelet suspension to $2x10^8$ platelets mL^{-1} with

Comment [JG17]: Nice to see the old methods still used…..takes me back!

Comment [JG18]: Please add to the hardware section of section 2 (probably don't need to use a specific make or model).

Comment [ST(oCaEM19]: Added to hardware section

Comment [JG20]: Not sure I understand this – you do a slow spin – then take most of the blood – including red cells into another tube and spin again?

Comment [ST(oCaEM21]: Yes – It does seem strange but I think it does a good job of removing most of the red blood cells, but ensuring that we recover the maximum number of platelets , as in mouse they sit on top of the red cells after spinning. I've modified the text slightly in this section to make it (hopefully) clearer.

Comment [JG22]: Is this the plasma and platelet layer from the last step combined (sorry for my pedantry ….I am trying to look at this from the novice's perspective.

Comment [ST(oCaEM23]: Added extra detail to clarify

modified Tyrode's buffer. Rest the platelets for 30 mins before starting the spreading experiments to allow excess prostacyclin to decay.

3.2 Sample preparation

3.2.1 Preparation of coverslips and dishes for platelet spreading

1. For STORM/PALM imaging 35 mm diameter, #1.5 (0.17 mm) glass bottomed ~~MatTek~~ dishes are used. For SIM imaging either 13 mm diameter, #1.5 (0.17 mm) glass coverslips or 35 mm #1.5 (0.17 mm) glass bottomed ~~MatTek~~ dishes are used. (Note 1)

2. Coat the imaging surface by incubating with 100 µg mL^{-1} of fibrinogen (in PBS) overnight at 4 °C.

3. Block any remaining uncoated glass by incubation with 5 mg mL^{-1} BSA for 1 h at room temperature followed by three washes with PBS. Store coated surface in PBS until ready for platelet spreading.

3.2.2 Spreading and labelling of platelet F-actin and phosphorylated proteins

1. Dilute washed and rested platelets to 2 x 10^7 platelets mL^{-1} in Tyrode's buffer and add to the glass bottomed dish or coverslip (held in a 12-well plate) and allow to spread on the coated surface for 45 min at 37 °C (Note 2).

2. After spreading, wash non-adhered cells away with PBS pre-warmed to 37 °C and fix the spread platelets with 10 % formalin solution for 10 min (Note 3).

3. Following fixation, wash coverslips three times in PBS. If aldehyde based fixation methods are used then residual fixative can be quenched by incubating the fixed platelets in 50 mM ammonium chloride for 10 min, followed by three PBS washes.

4. Permeabilise cells with 0.1 % Triton X-100 (v:v) in PBS for 5 min, followed by three PBS washes (Note 4).

Comment [JG24]: The publishers don't like suppliers in the actual methods section. Can you adapt moving the MatTek dishes to Note 1 as one of the options – where you say that a range of options are available.

Comment [ST(oCaEM25]: Removed supplier from here and included in Note section

Comment [JG26]: Please state temp

Comment [ST(oCaEM27]: Temperature added

5. If the platelets are going to be labelled with an antibody a blocking step needs to be performed to reduce non-specific antibody binding. Incubate the platelets in block buffer for 1 h at room temperature.

6. For SIM imaging of F-actin, incubate fixed and permeabilised platelets in a 1: 500 dilution of Alexa488-phalloidin in PBS for 1 h at room temperature (Note 5). Wash samples three times in PBS.

6. For dSTORM imaging of tyrosine phosphorylated proteins, dilute the 4G10 antibody 1:500 into block buffer and incubate with platelets for 1 h at room temperature (Note 6). Following labelling, wash three times with PBS.

Comment [JG28]: OK?

Comment [ST(oCaEM29]: Yes!

7. Dilute the anti-mouse AlexaFluor-647 secondary antibody 1: 300 into block buffer and incubate with platelets for 1 h at room temperature (Note 7). Wash samples three times in PBS.

8. For SIM imaging, mount coverslips onto glass slides using non-fluorescing aqueous mounting medium~~Hydromount solution~~ and store at 4 °C. Samples for SIM imaging in dishes can be stored in PBS at 4 °C.

Comment [JG30]: Nf this type of mounting medium is the only that work please leave in – if other possibilities exist remove 'hydromount'

Comment [ST(oCaEM31]: Updated with generic name

9. For dSTORM/PALM imaging, store dishes in PBS at 4 °C.

3.3 Super-resolution imaging of platelet samples

3.3.1 SIM imaging of platelet samples

For optimal imaging results using SIM, the microscope needs to be set up, aligned and calibrated correctly. For multi-user/core facilities, this should be done routinely by the facility, but for single user or non-facility based systems, this will need to be carried out by the user. The details of this calibration are beyond the scope of this chapter, but extensive details on the theory of SIM, how to perform calibrations and how to troubleshoot imaging problems have been provided by Demmerle *et al* [27].

1. Place the dish, or slide, to be imaged on the microscope stage within the microscope incubator. Using high NA immersion oil applied to the 100x lens, bring the objective lens up to engage with the

coverslip. Bring the cells into focus on the camera by using the fine focus and engage focus drift correction system ~~PFS~~.

2. In the software, select the required optical configuration and correct diffraction grating for your fluorophore(s). ~~(for Alexa488-phalloidin use 3D-SIM 488 & 100 EX V-R respectively) and set the laser fibre input to "Multi".~~

3. Adjust the ~~488~~ laser power and camera exposure time to achieve an image intensity signal level (grey levels) of approximately 4,000 (for the 14 bit camera setting used). (Note 8)

4. Select ~~3D~~2D-SIM options in ~~the capture menu~~your software, ~~-~~ensure that the grating pattern can be clearly seen in focus in the image (Fig 1A) and ~~press capture to~~ acquire a single z plane. This will collect a raw image which contains the 15 frames needed for SIM reconstruction (3 rotations and 5 phases – see Fig 1A). For 3D-SIM, Z-stacks through the cell can also be acquired by using the motorised stage of the microscope and capturing the multi-rotation, multi-phase images at each focal plane ~~setting the top and bottom plane of the cell and the z-step size in the "nd acquisition box"~~ (Note 9). A diffraction limited image can be collected for reference (Fig 1B & C). (Note 10).

3.3.2 Image processing of raw SIM data

~~1.~~ It is critical for optimal image reconstruction that the correct number of grey levels are collected and the grating pattern is seen with good contrast in the raw images. Further adjustments to the final reconstructed image quality can be made by adjusting reconstruction parameters in the software.

1. Open the raw SIM image data file within the respective microscope analysis package for your microscope system.

2. In a z-stack, remove the slices that are well above and below the area of interest to improve the quality of the reconstruction.

3. Most commonly used SIM reconstruction software packages allow adjustment of certain parameters to improve the final reconstructed SIM image. These may include noise filtering, out of

Comment [JG32]: If you tone down the Nikon specificity in earlier sections you might want to use a different term for this – "continuous automated focus system"….or something less made up by me!

Comment [ST(oCaEM33]: Have replaced with something made up by me!

Comment [JG34]: Is this Nikon specific?

Comment [ST(oCaEM35]: Yes – I have replaced with a generic statement

Comment [JG36]: Is this Nikon specific? Perhaps rephrase to make more generic?

Comment [ST(oCaEM37]: Probably easier to just say acquire!

Comment [JG38]: ditto

Comment [ST(oCaEM39]: I have simplified this statement

Comment [JG40]: this reads a bit like a pre-amble to the section rather than an instruction….

Comment [SGT41]: Agreed - have taken away the number and left as a pre-amble. Also made method below more generic and removed Nikon specific items.

focus blur suppression and illumination modulation contrast ratio. Details of how these parameters will affect image reconstruction will be found in your software's help files. Additionally, Demmerle et al [27] describe the theoretical principles behind these parameters in greater detail to enable the user to perform optimum, artefact free reconstructions. ~~Within the N-SIM window selecting the "Param" button allows a preview of the image to be generated following adjustment of parameters controlling 'Illumination modulation contrast' (use larger values where grating contrast is poor), 'high resolution noise suppression' (larger values help remove high frequency artefacts at the cost of fine detail structure) and 'Out of focus blur suppression' (use larger values to suppress out of focus light).~~

4. Once satisfied with the parameters click reconstruct to apply to the image and generate a SIM super-resolution image (Fig 1E & F)

5. A quick readout of SIM image ~~Image~~ quality can be performed using ~~assessed by performing~~ a Fourier transform of the ~~data~~ reconstructed image in Fiji [41, 42]. The~~to reveal the~~ characteristic petal shaped pattern of a good SIM image (Fig 1D & G)~~.~~ indicates that the ~~reconstruction was normal and~~ ~~the~~ reconstructed image contains effective information from each component in the raw data set. Deviations from this petal shaped pattern may indicate problems with one or more components of the reconstruction. ~~Fourier transforms can be carried out on the reconstructed image in Fiji [41,42]~~ (Note 11).

Comment [JG42]: Nikon specific?

Comment [JG43]: Very pretty – How do you interpret this – or use as a measure of SIM quality?

Comment [ST(oCaEM44]: Hopefull y now made this clear

3.3.3 dSTORM imaging of platelet samples

For optimal imaging results using dSTORM, the microscope needs to be set up, aligned and calibrated correctly (this includes TIRF alignment, 3D calibration files and chromatic aberration warp files for 3D and multi-colour STORM respectively). For multi-user/core facilities, this should be done routinely by the facility, but for single user or non-facility based systems, this will need to be carried out by the user. The details of this calibration are beyond the scope of this chapter, but extensive details on how to perform calibrations should be provide by the microscope provider and troubleshooting of STORM imaging problems have been provided by several groups [43-45,18].

1. Before imaging, pre-warm the microscope incubator to 28 °C for a number of hours (usually overnight). Place both the glass-bottomed ~~MatTek~~ dish with the sample and the STORM blinking buffer into the microscope incubator to warm for at least 15 min prior to imaging (Note 12).

2. Put the blinking buffer into the glass-bottomed ~~imaging~~ dish (Note 13) and place the dish onto the microscope stage within the microscope incubator, apply high NA immersion oil to the lens and bring up the lens to engage with the coverslip. Use fine focus to bring the sample into focus on the camera and engage focus drift correction system ~~PFS~~ (Note 14).

3. Using low laser power (usually less than <0.1 % to avoid bleaching the sample) identify an appropriate field of view (FOV), ~~and~~ focus the sample~~. Select a camera region of interest (ROI) of 256 x 256 pixels~~ and take a reference snapshot (Fig 2A). If a 3D image is required ensure that the cylindrical lens in in position and 3D-STORM mode is selected before starting acquisition.

4. Start a live image and then increase the power of the 647 nm laser to 100 % ~~(Camera exposure time; 1 frame (~10 ms), Conversion gain: 3, EM gain: 300)~~. Follow the live image until the sample has bleached and individual fluorescent blinks are observed (usually between 1 and 30 s depending on the label used). At this point start image acquistion and capture 20,000 frames using an appropriate exposure time and camera setting. (For the examples shown here we used a camera exposure time; 1 frame (~10 ms), Conversion gain: 3, EM gain: 300). Increase 405 nm laser power in ~2.5- 5 % steps every 30 s throughout the acquisition to assist in reactivation of fluoropohore from the dark state to maintain the number of detected blinks per frame. Numbers of molecules detected and the preview dSTORM image can be followed by selecting the appropriate software functions. At the end of acquisition save files into the appropriate file format for your software.~~ files are automatically saved as .nd2 files and tagged as STORM images by the software.~~

5. Return laser settings to starting values. Select a new FOV for imaging and repeat the above process.

3.3.4 Image processing of raw dSTORM data

Comment [JG45]: Cell culture dish? Actually – called imaging dish in next point

Comment [JG46]: As earlier

Comment [JG47]: Nikon specific?

Comment [ST(oCaEM48]: Made generic but added specifics for the images given as examples.

Comment [JG49]: Nikon specific?

1. Open the raw STORM image data file~~s (.nd2 file)~~ within the respective microscope analysis package for your microscope system. ~~the N-STORM module in NIS-Elements~~ (Note 15).

2. Select a middle frame from the image sequence and use this to set the threshold for point identification ~~through the "Identification settings" dialogue box~~. Different threshold values should be tested to ensure that all 'real' blinks are detected and that background is not (Note 16).

3. If the image is 3D (i.e. was collected using the cylindrical lens), then select the appropriate options in your software package ~~"fit overlapping peaks" option can be used~~ which will maximise~~s~~ the identification of distorted point spread functions. A valid 3D calibration file is needed for this processing to assign correct z positions (Note 17).

4. Once settings have been confirmed, start reconstruction of the STORM image by clicking the process, start button. Once finished the software will display a high resolution image of the data (Fig 2B). The display options and any subsequent processing. (e.g. density filtering, rendering, 3D visualisation, etc.) can be performed (Fig 2C – E).

5. ~~The software~~STORM analysis ~~software~~ software ~~uses~~ contains an autocorrelation drift correction algorithm to recognise and correct drift in the image due to stage movement. An example of this can be seen in Figure 3. Furthermore, if multicolour imaging is used, the software can correct for chromatic aberrations assuming a valid warp calibration has been performed.

6. A molecule list which contains all the information for each detected molecule in the image can be exported for further analysis (Note 18).

4. Notes

Note 1: ~~Other~~ gGlass-bottomed dishes or multiwell slides can be used for both SIM and dSTORM. e.g. Labtek multiwell slide, MatTek glass-bottomed dishes, etc. In addition, alternative materials are available (e.g. Ibidi slides) which have a similar refractive index to glass and so may also be considered for your application. If you use a different thickness coverslip (e.g. # 1 – 0.15 mm) you must remember to adjust the correction collar of the microscope to ensure that the point spread

Comment [JG50]: Remove? = Nikon specific

Comment [JG51]: "The respective microscope analysis package for your microscope system"

Comment [JG52]: Nikon?

Comment [JG53]: Nikon?

function is even. This can be tested by imaging a z-stack of fluorescent beads in the type of imaging dish you will be using. A good PSF has a symmetrical spread of the fluorescent signal as the z-series goes above and below the focal plane of the bead, when viewed in *xz* and *yz* orientation.

Note 2: Platelets can be spread for different time periods depending on the needs of your experiment. They can also be pre-incubated with inhibitors or antibodies to surface proteins of interest (which do not affect adhesion or spreading) as required. Ensure that appropriate controls for inhibitors are included in the experiments.

Note 3: The fixation method used will depend on the proteins/structures that you are interested in examining and will need to be determined empirically for your application.

Note 4: If you are using an antibody to an extracellular epitope permeabilisation of the cells may not be necessary. This will need to be determined for each antibody. One thing to be aware of if labelling after spreading is the potential for restricted antibody access to the epitope. Increased labelling at the cell edges may not be the real protein distribution but may represent inaccessibility of the antibody to areas that are tightly adhered. It is important therefore that antibodies are well characterised, for example in confocal microscopy first.

Note 5: Mice expressing Lifeact-GFP which labels F-actin [46] are available and these can be used to image F-actin dynamics in SIM in live platelets.

Note 6: Dilutions and incubation times will depend upon the antibody used and should be determined empirically for each antibody. It is possible to do dual labelling and it is good practice to optimise antibody labelling protocols for each antibody independently. Furthermore, samples which will be stored before imaging may benefit from post-labelling fixation [18].

Note 7: To increase spatial resolution, labelling cells with fluorescently conjugated antibody Fab fragments (~9 nm), or a camelid nanobody which is even smaller (~3 nm), is preferable to labelling with a primary antibody (~15 nm) followed by a fluorescently-tagged secondary antibody. This ensures that the fluorescent dye (which is detected) is as close as possible to the epitope of interest.

Using a primary plus secondary antibody approach can add up to 30 nm between the epitope and the detected 'blink' which, in turn, can artificially enlarge structures when viewed on the nanometre scale of dSTORM. Increasingly, samples with HALO and SNAP tags are being used for super-resolution imaging [47], but this application has not been extended into megakaryocytes or platelets to our knowledge

Note 8: It is worth spending time setting the appropriate exposure time and laser intensities at the beginning as this will affect subsequent image quality if it is not set correctly. A balance between laser power and exposure time needs to be obtained which gives a good dynamic range in the captured image whilst minimising photobleaching in the sample.

Note 9: In 3D-SIM, each z-plane consists of 15 individual images, therefore care must be taken to ensure that the cell does not bleach within the acquisition by limiting the laser power and the number of z-positions i.e. do not capture excessive images ~~take lots~~ above or below the focal plane. Bleaching will negatively affect image quality.

Comment [JG54]: "capture excessive images"?

Note 10: Multi-colour and live cell imaging is possible with SIM. Provided the microscope you use is equipped with the appropriate laser lines, several different fluorophores can be used to label multiple proteins of interest in both 2D and 3D. The use of s~~S~~ingle colour controls to ensure no bleed-through or cross-talk is occurring is recommended. Fluorescent labelling of platelets for live cell imaging could be in the form of a transgenic mouse model (protein of interest tagged with a fluorescent protein), a non-function blocking antibody to a surface receptor, or other fluorescent cell permeable probe. For these, labelling protocols will need to be determined by the user.

Note 11: ~~A suite of ImageJ plugins called SIMcheck [48] is available for checking the quality of SIM data and determining the resolution of SIM images.~~ If the characteristic petal shape is not seen when a Fourier transform is performed on your SIM images, acquisition settings ~~will~~may need to be adjusted to obtain a good raw image data set in which the grid pattern is clear. ~~,~~(e.g. adjusting focal plane, or ensuring that the exposure time and laser power are optimised to achieve the correct level of grey values). If the petal shape is missing one or more "lobes", this may indicate that there is a problem

with one or more of the diffraction grid pattern rotations or changes may need to be made to the reconstruction settings [27]. In addition, a suite of ImageJ plugins called SIMcheck [48] is available for checking the quality of SIM data and determining the resolution of SIM images.

Note 12: Having a stable temperature to image in is very important to minimise thermal drift which can have a big impact on STORM image quality. Heating the incubator for at least 12 h prior to imaging will ensure that drift is minimised. Setting the temperature to slightly higher than room temperature (e.g. 28 °C) will allow the incubator to maintain a steady temperature more easily.

Note 13: Depending on the fluorophore being imaged, the buffer conditions may need to be varied. Much work has been done on identifying the optimal buffers for different fluorophores [43,18] and so the appropriate combination of buffers for the experiment should be determined by the user.

Note 14: If your protein of interest is membrane-bound, for example a cell surface receptor, it is recommended that you image your spread platelet sample in TIRF mode. This will ensure that you are only illuminating, and therefore imaging, the proteins that are at the adherent platelet surface. This will also increase the signal to noise ratio which will result in cleaner images. For a detailed method on performing TIRF see Poulter *et al* [49].

Note 15: Raw data can alternatively be reconstructed using the Thunderstorm plugin [50] for Fiji [41].

Note 16: Individual software programme have different tools for performing this task. In the examples given in figure 2, the Nikon ~~The~~ "peak intensity" tool can help the user to identify the threshold values for a particular image. When this tool is placed over an area which you consider to be a blink, making sure the centre is over the brightest pixel, the intensity over background is displayed. This value can then be used to select the threshold level.

Note 17: ~~The "fit overlapping peaks" tool uses t~~The DAOSTORM algorithm is commonly used to fit overlapping blinks [51]. This is a useful feature especially for 3D data but it must be noted that this function will increase reconstruction processing time considerably.

Comment [JG55]: generic or Nikon specific?

Note 18: The resolution of the final super-resolved image can be determined using a number of methods. For SMLM data, the user can compare localisation precision (also known as uncertainty) for each identified molecule in the sample or measure full width half maximal (FWHM) distances of identified objects. Whilst these provides a good measure of the quality of the data generated, the effective resolution of the final image can be calculated using the Fourier Ring Correlation (FRC) method [52] which takes into account uncertainty and labelling density.

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Figure legends

Figure 1 – Structured Illumination Microscopy (SIM) of platelet actin cytoskeleton. A) Acquisition of 3D-SIM images on the N-SIM system generates an .nd2 file containing 15 images for each z position. These 15 images differ in the rotation and phase of the diffraction grating. The 3 images show examples of the 3 rotations (indicated by lines in bottom right corner). The diffraction grating pattern can be seen as striping within the image. B) Example diffraction limited epifluorescence image of a single z slice. C) Intensity profile from the line shown in panel B. Note that overall shape of cell is seen, but individual structural detail of the cytoskeleton is not. D) Fourier transform of the image shown in panel B showing the frequency space representation. Low frequency information is located in the centre. The size of the central observable region is defined by the maximum frequency the objective can transmit. E) Reconstructed SIM image of the same z slice as panel B. The increased contrast and resolution of the image is clear. F) Intensity line profile from the line in panel E. Note the increased resolution and contrast of the image results in individual actin bundles being resolved. G) Fourier transform of the image shown in panel E showing the characteristic petal shape indicating that the high frequency information from outside the diffraction limited observable region was successfully captured. Scale bar in all images = 5 µm.

Figure 2 – direct Stochastic Optical Reconstruction Microscopy (dSTORM) of platelets A) Diffraction limited epifluorescence image of a human platelet spread on fibrinogen and immunostained for phosphotyrosine. A secondary antibody conjugated with Alexa 647 was used. B) Reconstructed 2D-dSTORM image of cell in panel A. The increased resolution allows the resolution of diffraction limited structures as indicated by the arrow. C) Metrics provided by the reconstruction algorithm include total number of molecules detected, localisation precision (in xy for each molecule detected) and the number of photons counted per molecule. The lower graph shows the important relationship between photon count and localisation precision, with the most highly localised molecules being the ones with the highest photon count. D) Example of a 3D-dSTORM reconstructed image showing z position indicated by a colour scale. E) Zoomed in 3D representation of the box in

panel D showing 3D organisation of phosphotyrosine within the platelet. Scale bar in all images = 2.5 µm.

Figure 3 – Example of software based drift correction in final dSTORM images. A) Example of a reconstructed dSTORM image which exhibited some stage drift during acquisition prior to applying drift correction. B) The same image following application of the autocorrelation drift correction algorithm within NIS-Elements. Scale bar = 2.5 µm.