Special Report

Super-resolution microscopy in the diagnosis of platelet disorders

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Summary

Platelet granule deficiencies cause a number of disorders of blood clotting. The exact diagnosis or phenotyping of patients requires imaging of the platelet structure; however this has traditionally required the use of electron microscopy (EM) because the granules are too small to be resolved by conventional light microscopy. EM facilities are expensive and not widely available in hospitals, so an alternative approach would be highly beneficial. So-called “super-resolution” light microscopies offer a potential solution to this problem. In a recent study we demonstrated that one form of super-resolution microscopy – structured illumination microscopy (SIM), is able to reliably discriminate between platelets from patients with a dense granule disorder and those from healthy controls. This approach could be extended to related conditions.

[120 words]

Keywords

Super-resolution microscopy;

Structured Illumination Microscopy;

Platelet disorders;

Dense Granules;

Hermansky-Pudlak Syndrome

[5-10 keywords]

Abbreviations

EM	Electron Microscopy
SIM	Structured Illumination Microscopy
PALM	Photoactivation Localisation Microscopy
(d)STORM	(Direct) Stochastic Optical Reconstruction Microscopy
STED	Stimulated Emission Depletion
RESOLFT	Reversible Saturable Optical Fluorescence Transitions
MSIM	Multifocal SIM
ISIM	“Instant” SIM
NLSIM	Non-Linear SIM

Body of the article

Platelet Structure and Function

Platelets are circulating cell fragments which are best known for their critical role in haemostasis, although they perform a variety of other functions [1,2]. In humans, platelets are typically 2 µm to 5 µm in diameter and have a discoid shape due to the presence of a peripheral ring of microtubules [3]. They are produced by budding from megakaryocytes and are described as cell fragments because they do not contain a nucleus. Instead platelets contain a set of specialised organelles dedicated to their various functions (illustrated schematically in Figure 1).

These include a number of types of storage vesicles (also known as granules) whose contents are released into the plasma on platelet activation. They are traditionally

divided into dense (or δ-) granules and α-granules [2]. Recent work has suggested that this binary division is overly simplistic, identifying an additional granule type [4] and apparent subdivisions within α-granules [5]. Dense granules get their name for their appearance in whole mount electron micrographs; that is to say that they are electron-dense (due to the presence of a high concentration of calcium).

A variety of platelet disorders – collectively known as storage pool disorders - are known to be associated with deficiencies in platelet granules; α-granule deficiencies in α storage pool disorder (grey platelet syndrome); dense granule deficiencies in δ-storage pool disorder, Chediak-Higashi and Hermansky-Pudlak syndromes; and deficiencies of both types in αδ-storage pool disorder [2]. The deficiencies in these secretory granules result in a corresponding reduction in the release of their contents during platelet activation, leading to an impaired clotting response.

In many cases, to correctly diagnose these disorders, electron microscopy is required to elucidate the structural defects in the platelets, but is often not available. Various functional assays are available, but these do not identify the structural deficit underlying a platelet disorder. Apart from the requirement for the instrumentation and the sample preparation requirements, skilled interpretation of the images obtained is required, as the dense granules appear on a complex background that would challenge automated image analysis [6,7]. Furthermore the samples must be imaged on the day of preparation [DOI:10.1055/s-2007-995836]. To improve the specificity of diagnosis for these patients, it would be useful if an alternative approach could be developed that would be more widely available.

Limits of resolution

An obvious alternative to electron microscopy would be to use some form of light microscopy. In particular, fluorescence microscopy would allow staining for specific markers that would allow structures of interest within the platelet to be visualised and counted. However, platelets are only 2 µm to 5 µm in diameter; dense granules are 150 nm and alpha granules only 200 nm to 400 nm [8]. Since the resolution of conventional light microscopy is only ~200 nm to 250 nm, platelet granules cannot be visualised with sufficient clarity to permit accurate quantification and diagnosis using such a microscope (see Box 1). A potential solution to this conundrum is the newly developed family of super-resolution microscopies, which are fluorescence microscopes that can achieve resolutions two- to ten-fold higher than conventional light microscopy (Box 2).

Super-resolution imaging of platelets

In a recent study, we set out to demonstrate the principle of using super-resolution microscopy to diagnose platelet granule disorders [7]. From the methods listed in Box 2, we chose to evaluate the use of SIM, because the resolution (~120 nm) is sufficient for our purposes and the method is fast enough to be practical in a diagnostic scenario. As can be seen from the example images shown in Figure 2, SIM not only improves the resolution of the images, but also the contrast, providing much clearer images that are readily suited to automated analysis.

One of the challenges we encountered is that there is currently no well-established and unique and specific protein marker for which antibodies are widely available that can be used for dense granules. We therefore chose to use as marker the integral

membrane protein CD63 which has previously been found to be correlated with dense granule deficiency [9].

Hermansky-Pudlak syndrome is a well-studied disease which in addition to dense granule deficiency is associated with a range of other symptoms including albinism that are believed to be related to deficiencies in the formation of vesicular organelles including melanosomes and lysosomes [10]. The genetics of the disease is also fairly well understood; 10 genes have been identified to date [OMIM #203300 http://omim.org/entry/203300] [11]. It therefore makes an ideal test case for this new diagnostic approach, as an unambiguous diagnosis can be made.

To evaluate the use of super-resolution microscopy in the diagnosis of this condition, we analysed samples from 7 healthy controls and 3 patients with different forms of HPS [7]. As a reference point, we first imaged the samples using the current gold standard whole mount electron microscopy approach. We found that the control platelets had an average of 3.5 dense granules, whereas very few platelets from the HPOS patients contained any dense granules (average 0.07).

The platelets were purified and attached to a cover slip at a moderate density to facilitate the collection of adequate statistics. The platelets were stained with antibodies to CD63 and tubulin. Visual inspection of the resulting images shows how the tubulin ring acts as a clear marker for the platelet periphery. Multiple CD63-positive organelles are clearly seen within each platelet. Differences between the HPS and control samples are readily apparent; in the HPS patients there are fewer CD63 positive organelles; the staining is fainter; and there appears to be additional CD63 at the platelet periphery.

These impressions were confirmed by an automated image processing pipeline and statistical analysis, which we used to count the number of organelles and make comparisons between the control and HPS samples. We found that the controls had an average of 6.8 CD63 positive structures per platelet, whilst the HPS patients had only 2.4. While these numbers are higher than seen for the EM approach, this was expected as CD63 is known not to be confined to dense granules. The use of the automated analysis meant that the control and patient samples could be distinguished with a 99% confidence level. We feel that this shows the power of the super-resolution imaging approach. (put this in later?)The ease of differentiating patient from control, plus the potential low cost and possibly centralised facility of this approach giving a low threshold for usage, could allow for shortening the time to definitive diagnosis for this rare disease. Furthermore, this imaging approach could be extended by the use of additional markers to create a detailed profile of platelet structures that could be used for diagnosis of a wide range of platelet disorders. For example, by adding a marker for α-granules it would be possible to identify a range of disorders including grey platelet syndrome and αδ-storage pool disorder.

A recent paper used a different super-resolution technique, STED (see Box 2) to image the distribution of a number of proteins which are all believed to be located in the α-granules [12]. STED has a higher resolution than SIM but is typically somewhat slower {ref?}. Interestingly, with the higher resolution microscopy it was shown that these proteins had different distributions, suggesting that these proteins were localised to sub-compartments within the α-granules. This reinforces our view that the detailed structural information obtained by super-resolution microscopy is an important tool for understanding platelet function and potentially for diagnosing a variety of disease

associated states of the platelet. In particular, the markers studied show the potential for investigating aspects of platelet function beyond clotting.

Future prospects

We believe that the papers described above show that super-resolution microscopy has some unique advantages as a method for investigating platelet structure and function, and potentially diagnosing structural platelet disorders. Interestingly another recent paper described the use of super-resolution microscopy in the diagnosis of kidney disorders [13]. As with the diagnosis of platelet granule deficiencies, electron microscopy is normally required to diagnose nephrotic disease, where the structure of the podocyte cells of the glomerulus may be disturbed. However here the process is even more challenging, requiring a kidney biopsy and embedding and thin sectioning of the sample. Pullman *et al.* show that SIM is valuable here too, with less extensive sample preparation requirements giving a faster result and also the ability to image at high resolution in 3D. Taken together with the work on platelets described above, it seems likely that there is a niche of applications in disease diagnosis and pathology where super-resolution microscopy can replace electron microscopy, potentially reducing costs and increasing the level of detailed information available to clinicians.

Of course it is true that the first commercial super-resolution instruments were rather expensive (up to $250,000 this is very low end! Zeiss began at £1M.) – but costs are likely to continue to come down rapidly, and we anticipate the introduction of a new generation of low-cost super-resolution devices (although visitech are 200K+). SIM is a technology which seems particularly suited to diagnostic applications because of its relatively high throughput, its ease of use, and a lack of technical obstacles to the production of relatively inexpensive instruments. Another advantage of SIM, at least

for it's application to platelets, is that samples can be fixed and imaged days or weeks after preparation, so that not very hospital or clinic would require its own instrument.

Other advantages of SIM over electron microscopy include the facility with which multiple markers can be imaged in parallel, the ability to image structures in 3D at high resolution without sectioning, and the ease of automating the analysis of immunofluorescent images.

For platelet disease in particular, it is likely that genetic testing will be used increasingly in diagnosis of the hereditary platelet disorders. However, as new loci are still being discovered [11] and the effects of mutations vary between individuals, determining phenotype as well as genotype will continue to be important for the foreseeable future.

Expert commentary

While super-resolution microscopy has rapidly achieved awareness and increasing adoption in the cell biology and neurobiology research communities, and in related fields, there is as yet little awareness of these methodologies and their potential applications among clinicians. We feel that their potential is clear, but much more work needs to be undertaken to demonstrate their utility in the clinic. In particular, those niche areas where a subcellular structural detail is invaluable for phenotyping and potentially diagnosis and/or stratification should be more fully explored. Different super-resolution techniques will be applicable in different scenarios. Here we adopted SIM because although it does not give the highest resolution, it is adequate to quantify platelet granules and rapidly acquired high-contrast fluorescence images. Blood cells are a particularly attractive target for super-resolution imaging because they are

relatively easily obtained and sample preparation is more straightforward than other tissue types.

[The author's expert view on the current status of the field under discussion.]

Five-year view

Although super-resolution microscopes are currently expensive, we anticipate that lower cost systems will soon become widely available. This will enable applications beyond pure research, including their use in the diagnosis of some types of disease. The rich information that super-resolution methods can provide on disease phenotypes at a sub-cellular level will complement the increasing availability of genomic data for patients.

Key issues

- Structural platelet disorders often involve deficiencies in certain types of granule (organelle) within the platelet.
- The rarity of these disorders leads to slow initial diagnosis.
- Because platelets are typically 2 µm to 5 µm in diameter, and the dense granules are approximately 150 nm, conventional light microscopy cannot accurately measure the number of organelles within a platelet. Therefore electron microscopy has been required to be able to image them.
- Structured Illumination Microscopy can resolve these structures. Although its resolution is lower than EM, it is sufficient to accurately count granules and has the potential to be extended to observe multiple markers simultaneously, and therefore enabling the diagnosis of multiple types of granule deficiency.

- The high contrast, and the presence within the platelet of the distinctive ring of microtubules, facilitates the automation of the counting process, giving very statistically robust discrimination between patients and controls. This would be much more challenging with EM images
- [Statistics – robustness]
- Hermansky Pudlak – good demonstration, but work needs to be extended to other platelet disorders
- Applicability to other markers/diseases – multiple markers

[8–10 bullet points summarizing the review.]

References: target of 50 references

Reference annotations

[Please highlight 6–8 references that are of particular significance to the subject under review as "* of interest" or "** of considerable interest" and provide a brief (1–2 line) synopsis.]

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Figures

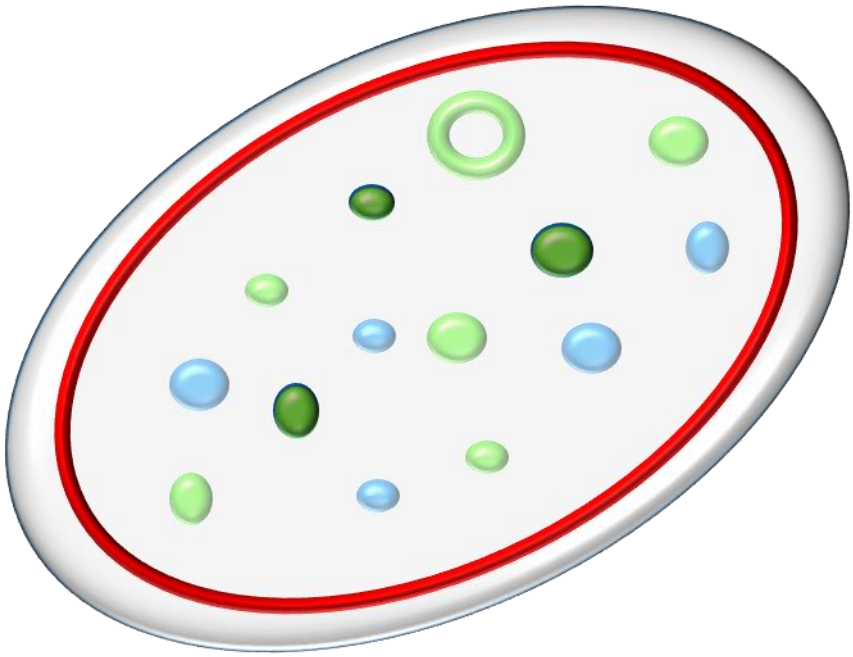

Figure 1 Schematic of Platelet Structure

A simplified schematic of the key structural features of the platelet relevant to this article. The platelet membrane, shown in pale grey, encloses internal structures

including the tubulin ring (red); α-granules (pale blue); dense granules (dark grey) and $CD63^+$ structures (pale green). Note that all dense granules are $CD63^+$ but that other structures are also $CD63^+$; there is no unique marker for dense granules. Structures not shown for clarity include actin filaments, mitochondria, lysosomes, peroxisomes, T-granules and the canalicular system.

Figure 2 Comparison of diffraction-limited and super-resolution images of a platelet

Tables

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Boxes

Box 1 What is Super-resolution Microscopy?

- Ernst Abbe showed that the resolution of an image in light microscopy has a theoretical limit – this is approximately half the wavelength of the light. For visible light this equates to approximately 200 nm to 250 nm [14,15].
- This limit is caused by the diffraction of the light as it propagates through the microscope. There is no way to stop this diffraction, so it was thought that this resolution limit – sometimes called the "diffraction limit" was a fundamental limit that could not be overcome.
- However, recent decades have seen the development of at least three independent ways of by-passing the limit and they are beginning to make a significant impact in cell biology and related fields of research [16] – see Box 2.
- The importance of these innovations was recognised by the awarding of the 2014 Nobel Prize in Chemistry to some of the field's pioneers: Eric Betzig, Stefan W. Hell and William E. Moerner [17,18]

Box 2 Super-resolution microscopy methods

There are many super-resolution techniques, which are typically variants of three main approaches. These are summarised below:

Localisation microscopies

Although the resolutions typically obtained in light microscopy (~250 nm) are much larger than molecules, individual fluorescent molecules can still be imaged and their positions localised with much higher precisions (~10 nm). In the localisation microscopies, this process is repeated many thousands of times to map out the positions of all the fluorescent molecules. This information can then be used to

reconstruct a super-resolution image. Localisation microscopy requires some way of "switching" the fluorescence of the target molecules, and the need for many cycles of switching and imaging means that this approach is relatively slow. Examples include PALM and STORM and their variants.

Point Spread Function Engineering

The shape of a diffraction limited focal spot in a microscope is referred to as the Point Spread Function. By manipulating this point spread function, higher resolutions can be obtained. Variants include STED, RESOLFT, and conical diffraction.

Structured Illumination Microscopies

By using a series of patterns of illumination, and processing the resulting images mathematically, it is possible to improve spatial resolution – typically by a factor of 2 (~120 nm). The illumination patterns are typically of lines (a sinusoidal grating) or points. Because only a few images are often needed, this can be a relatively fast method. Variants include SIM, MSIM, ISIM, NLSIM and others.