Reflectance imaging for visualization of unlabelled structures using Nikon A1 and N-SIM

Reflectance imaging describes a subset of light microscopy methods that permit the formation of an intensity image from light that is back-scattered from a sample. This allows reflectance imaging to be performed alongside techniques such as fluorescence imaging, or as a standalone methodology for the investigation of a variety of labelled and unlabelled samples.

Interference Reflectance Microscopy (IRM) was the initial application of reflected light imaging[1]. IRM involves the formation of an image from signal that is generated by light interacting at the boundary of substrates with differing refractive indices (RI). Since this original application, a variety of different configurations have emerged leading to the generation of images based on RI mismatches that are naturally occurring throughout cell and tissues samples. However, contrast can also be artificially induced by the introduction of materials with high RI and optical density, analogous to fluorescent labelling.

The Nikon A1 point-scanning confocal and the Nikon N-SIM super-resolution structured illumination system are both capable of imaging in reflectance mode; each technique offering different advantages and disadvantages for critical investigations into reflectant substrates, such as nanoparticles (NPs), which have previously been discussed[2,3]. Other modalities can also benefit from the use of reflectance configuration to provide structural information, including multiphoton *in vivo* investigations[4].

Reflectance imaging: introduction

Contrast is generated in microscopy images through the interaction of light with different specimen components. In traditional fluorescence microscopy this signal is generated through the emission of light from excited fluorophores, conjugated to molecules of interest. However fluorophores can be difficult to attach, may alter physicochemical properties and cellular processing of labelled substrate, can detach during experiments, and are subject to photobleaching[5,6].

In transmission and reflectance imaging contrast is generated by the interaction of light at boundaries present within the sample. These boundaries are present at the regional interfaces where RI differs; in transmitted light microscopy it is the light that is transmitted through that is collected, whereas in reflectance microscopy it is the back-scattered light that forms the image. Reflectance offers several advantages in terms of sample interrogation:

1) No labels are required to visualize various components within the sample as with fluorescence imaging
2) Photobleaching does not occur as in fluorescence imaging
3) The Signal-to-Noise Ratio (SNR) of objects of interest is greatly enhanced compared to transmission microscopy

Therefore reflectance imaging becomes the obvious choice for the investigation of samples that contain objects of interest with substantially different RI's or optical densities to their surroundings – such as the imaging of intracellular metallic NPs[2,3,7,8].

Reflectance imaging using the Nikon A1 confocal system

One of the initial limitations of reflectance imaging is the high level of background present in highly scattering environments, such as cells. Confocal microscopy provides a means to increase both SNR and resolution compared to epi-illumination in reflectance mode by filtering out-of-focus light through the conjugate pinhole system. This also permits optical sectioning. Confocal reflectance images can be acquired using a confocal microscope such as the Nikon A1 by simply changing the dichroic to the transmitted / reflectance mirror (BS20/80) and set up of the 4th channel with the laser wavelength of choice (e.g. 488 nm) (**Figure 1**) (Guggenheim *et al.*, 2016).

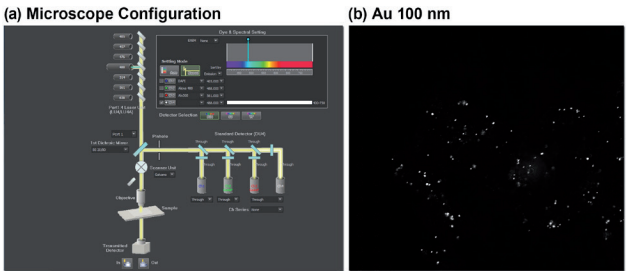

Figure 1. Example of the microscope acquisition configuration and an example reflectance confocal image. A) The configuration is set up using the fourth channel, using the 488 nm laser. The dichroic is set to BS20/80 and all light paths are set to 'through' as seen on the image. This allows the acquisition of images such as those shown on B) of cellular internalized 100 nm gold nanoparticles (grey).

Dr. Emily J Guggenheim[1] and Dr. Joshua Z Rappoport[2]

1. *School of Geography Earth and Environmental Sciences, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK*
2. *Center for Advanced Microscopy and Nikon Imaging Centre, Feinberg School of Medicine, Northwestern University, Chicago IL, 60611, USA*

Reflectance imaging of unlabelled samples is possible as long as the structure of interest has sufficiently different RI relative to the surrounding media[1,9]. Alternatively, light reflective probes, such as the NPs seen in Figures 1 – 4, can be applied to biological specimens, akin to the labelling of subcellular components with fluorophores. These agents can introduce strong contrast at specific locations within the sample[2,8,10].

Due to increases in the use of NPs in commercial and biomedical applications, and the associated lack of available safety and mechanistic data, these types of NPs can often be the main focus of bio-medical investigations. Metallic NPs give rise to significant contrast using reflectance imaging and Reflectance Confocal Microscopy (RCM) is ideal for the investigation into the behaviour of these NPs. This is partly because of instrument accessibility, and partly due to the potential to combine these studies with fluorescent labelling, such as endo/lysosomal staining to determining trafficking route and fate (**Figure 2**)[3]. Therefore the intracellular localization, trafficking and toxicity of NPs such as these have been investigated using reflectance imaging, in cell and animal models. Examples of these NPs are shown in Figure 2[2,3,8,10,11]. Figure 2 also shows an example of how RCM can be used to image 3D cellular structures, such as spheroids, in an attempt to determine and/or increase cellular uptake of NPs that may prove beneficial in future cancer therapy strategies.

RCM can provide semi-quantitative results, particularly beneficial within the field of nanoresearch when combined with automated processing and analysis to increase the throughput of studies[2,3]. However, in order to get absolute quantitative results, efforts have been made to perform correlative experiments by the incorporation of modalities such as Transmission Electron Microscopy (TEM) and Mass Spectrometry (MS) into workflows[2,11].

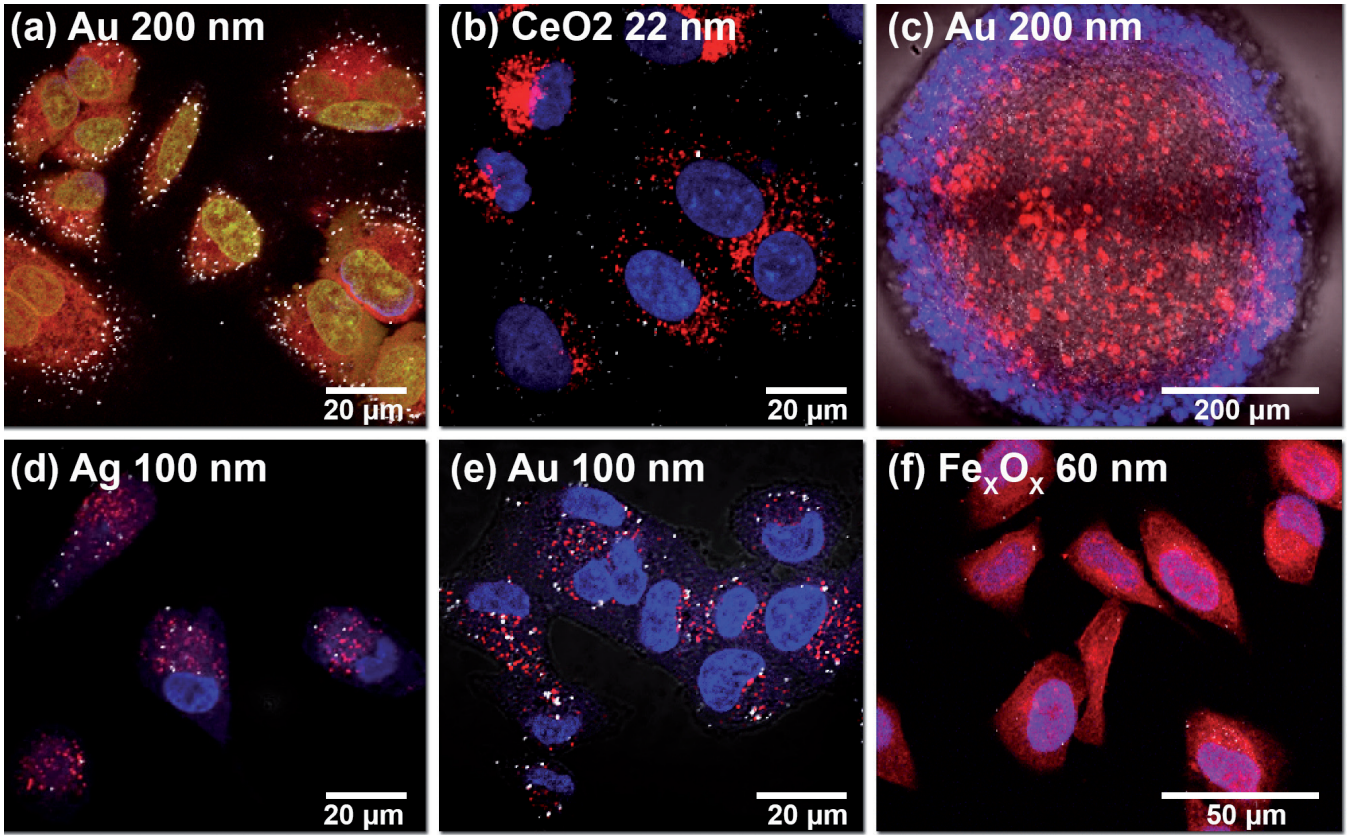

Figure 2. Example of confocal reflectance and fluorescent imaging of different metallic NP. The images show NP uptake into cells or cell spheroids of the following A) Au 200 nm B, B) CeO2 22 nm C) Au 200 nm, D) Ag 100 nm, E) Au 100 nm and F) carboxydextran coated iron oxide (Fe_2O_3 and Fe_3O_4) 60 nm, all depicted in grey. Nuclear staining is achieved using blue DAPI staining in all images. The cytoplasm is stained using CTO (orange/red) in (a, c, f). Lysotracker (red) is used to identify the lysosomal localization of different NPs in (b, d, e). The top right image shows a large image capture of a 500 μm diameter spheroid treated (in suspension) with gold NP (grey). The view is a maximum projection of a Z-stack that captures 40 μm of the spheroid from the coverslip.

Reflectance imaging using the Nikon N-SIM super-resolution system

Despite the advantages that confocal reflectance offers, the fundamental restrictions on resolution that govern conventional light imaging modalities still apply. Therefore increasing effort is being placed on the ability to acquire images with resolutions that surpass the diffraction limit of light, collectively termed super-resolution techniques. Structured Illumination Microscopy (SIM) can increase the spatial resolution two-fold with relatively small loss in temporal resolution, rendering it advantageous for live cell imaging[12]. Despite the widespread use of SIM in fluorescence imaging, the use of SIM in reflectance mode is not often documented.

In a traditional Nikon N-SIM acquisition, a filter cube is present within the light path consisting of an excitation filter, emission (or barrier) filter and a dichroic mirror. This allows light emitted from the specimen to pass through and reach the detector, while also blocking the detection of the excitation light. When performing Reflectance SIM (R-SIM), the filter cube is replaced with a half mirror that permits the transmission of the light reflected from a sample at the same wavelength as the incident light. In R-SIM the sample is illuminated in the same manner as for fluorescent SIM, whereby a grating is used to structure the incident light allowing acquisition of several images that can then be reconstructed in the Fourier domain to yield the super-resolution image[12]. This method has been applied to the investigation of intracellular NPs using both custom and commercial microscopes[2,7].

The ability to resolve structures separated by less than the diffraction limit by label-free light microscopy offers significant advantages for localising the NP clusters with increased precision and accuracy. NPs with varied compositions appear to be detected, albeit with different efficiency using R-SIM (**Figure 3**). For example, superparamagnetic iron oxide nanoparticles (SPIONs) appear to be better detected by RCM than R-SIM (**Figure 4**)[2]. Lower detection with R-SIM is a result of the wide-field illumination method used, and therefore high levels of background scattering. R-SIM can therefore be a particularly useful source of additional resolution and information within biological investigations, due to the minimal sample preparation necessary and, like with confocal, ease of combination with fluorescent labelling. Therefore R-SIM with the Nikon N-SIM instrument can offer more certainty in studies such as colocalization assessment of NPs and labelled structures (**Figure 4**). The sensitivity and specificity of reflectance methods can be confirmed by using correlative workflows that include TEM, as done in previous studies[2].

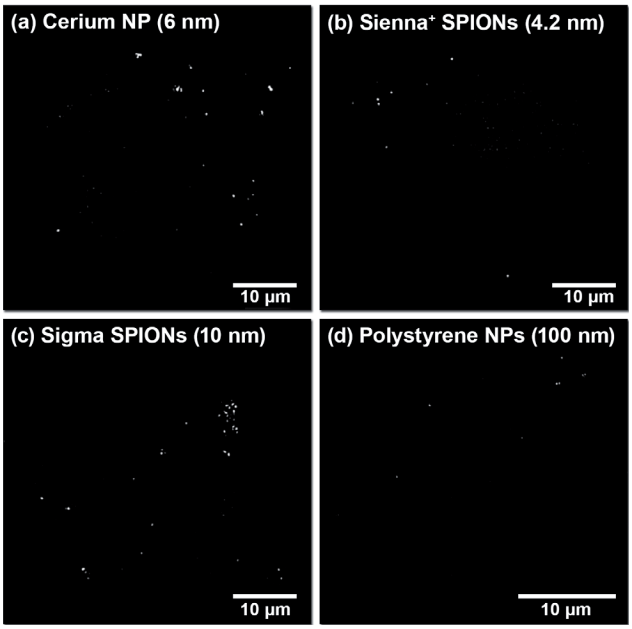

Figure 3. Different NPs are detected to different extents with R-SIM. For example CeO2 (a) and iron oxide (Fe_2O_3 and Fe_3O_4) 60 nm (b, c) are detected well, whereas polystyrene are not detected with as much sensitivity (d). All NP are depicted as grey.

Conclusions

Different illumination and scatter collection configurations can be employed to perform reflectance imaging; these include interferometry, confocal, evanescent wave, structured and oblique angle illumination. This facilitates a wide range of possible studies, particularly within the field of nanoresearch, where reflectance investigations will be instrumental in determining the safety and efficacy profiles of NPs for various applications. As different components of cells and tissue have different RIs, reflectance imaging can be advantageous for probing the structure and organisation of biological samples. The power of reflectance for providing additional and complimentary structural information is particularly pronounced in the field of *in vivo* imaging, contextualising existing diagnostic and monitoring strategies.

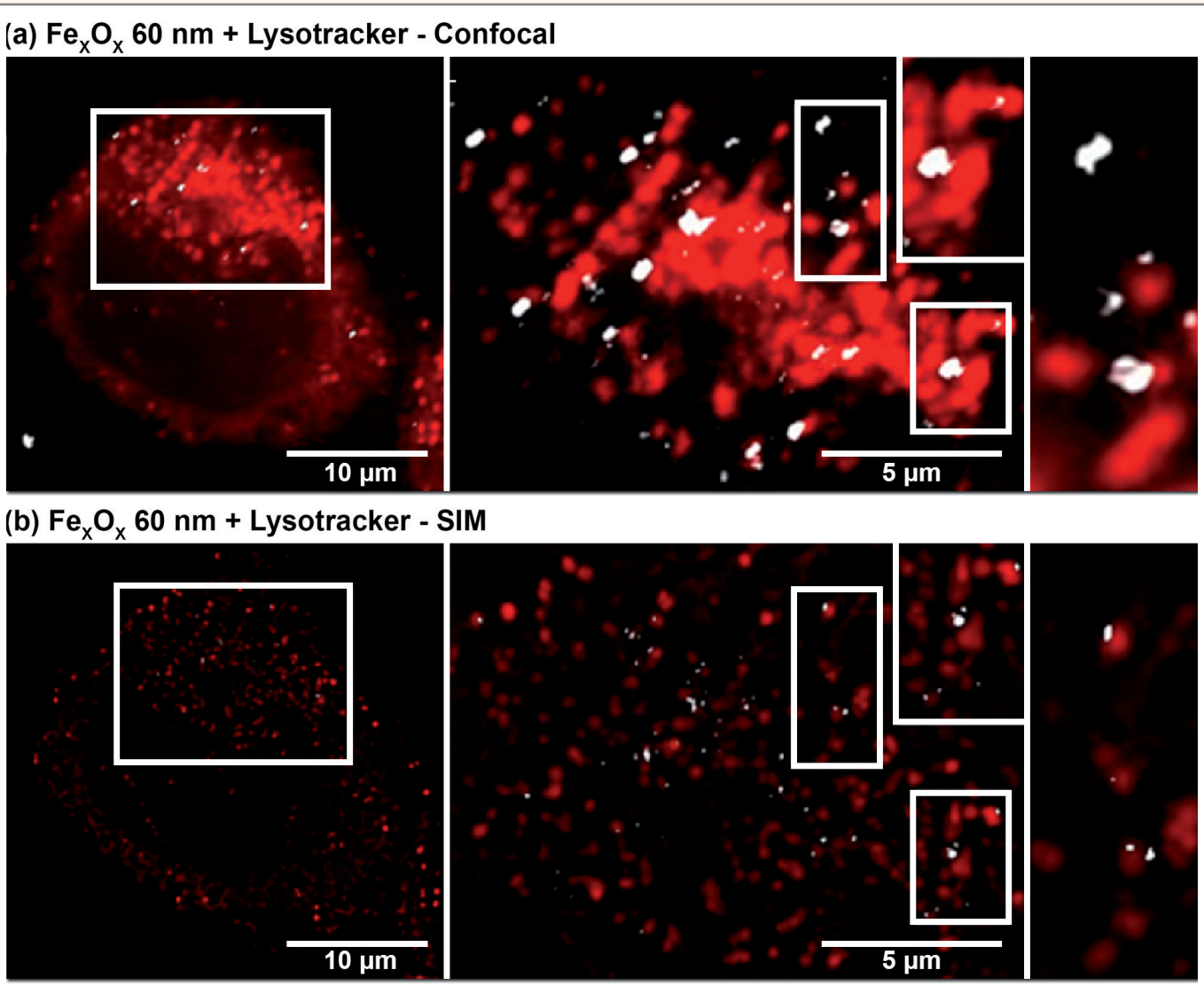

Figure 4. RCM and R-SIM of SPIONs (Fe_2O_3 and Fe_3O_4) 60 nm. NP signal in grey and lysosome signal in red. R-SIM leads to a substantial increase in resolution when compared to RCM for performing colocalization studies.

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