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Correlative super-resolution fluorescence and electron microscopy using conventional fluorescent proteins *in vacuo*

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ABSTRACT

Super-resolution light microscopy, correlative light and electron microscopy, and volume electron microscopy are revolutionising the way in which biological samples are examined and understood. Here, we combine these approaches to deliver super-accurate correlation of fluorescent proteins to cellular structures. We show that YFP and GFP have enhanced blinking properties when embedded in acrylic resin and imaged under partial vacuum, enabling *in vacuo* single molecule localisation microscopy. In conventional section-based correlative microscopy experiments, the specimen must be moved between imaging systems and/or further manipulated for optimal viewing. These steps can introduce undesirable alterations in the specimen, and complicate correlation between imaging modalities. We avoided these issues by using a scanning electron microscope with integrated optical microscope to acquire both localisation and electron microscopy images, which could then be precisely correlated. Collecting data from ultrathin sections also improved the axial resolution and signal-to-noise ratio of the raw localisation microscopy data. Expanding data collection across an array of sections will allow 3-dimensional correlation over unprecedented volumes. The performance of this technique is demonstrated on vaccinia virus (with YFP) and diacylglycerol in cellular membranes (with GFP).

1. Introduction

Life science imaging techniques have seen a number of revolutionary advances in recent years. Super-resolution light microscopy now allows visualisation at resolutions beyond the diffraction limit of light, and new electron imaging systems based on the scanning electron microscope (SEM; such as FIB SEM, SBF SEM, and array tomography) can deliver 3-dimensional cell and tissue ultrastructure through unprecedented volumes (Peddie and Collinson, 2014). At the same time, the practical application of correlative workflows that directly link protein localisation to cell ultrastructure has increased rapidly. Examples of protocols that correlate super-resolution light and electron microscopy from separate instruments include photoactivated localisation microscopy (PALM) with TEM and SEM (Betzig et al., 2006; Chang et al., 2014; Kopek et al., 2013; Sochacki et al., 2014; Watanabe et al., 2011), and stochastic optical reconstruction microscopy (STORM) with SEM (Loschberger et al., 2014; Wojcik et al., 2015). However, combining data from separate imaging systems can limit interpretation, as many techniques rely on the use of specific imaging conditions and probes that are not necessarily compatible with high precision correlation to electron microscopy data.

Probes are a key component in super-resolution correlative imaging. The GFP family, a ubiquitous tool in bioscience research, can be preserved through processing into the resin polymers that are necessary to support the specimen during electron imaging *in vacuo* (Bell et al., 2013; Johnson et al., 2015; Kukulski et al.,

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2011; Nixon et al., 2009; Peddie et al., 2014; Watanabe et al., 2011). We recently showed that YFP and GFP are photostable when embedded in acrylic resins using an in-resin fluorescence (IRF) protocol. Conservation of the hydration state of the FPs was critical for stable, long-lived fluorescence, and also for imaging ultrathin IRF sections *in vacuo* using an integrated light and scanning electron microscope (ILSEM) (Brama et al., 2015; Peddie et al., 2014). Fluorescence and electron images of ultrathin IRF sections are unique in that the information from both imaging modalities is gathered from the same physical slice (50–200 nm thick). As such, the axial (z) resolution corresponds to the physical slice thickness and the fluorescence images taken from IRF sections are inherently better than the diffraction limit. However, the lateral (xy) plane will still be diffraction limited if a widefield fluorescence (WF) microscope is used, so structures below ~200 nm cannot be resolved. Intriguingly, it was recently shown that standard FPs blink in IRF sections when imaged using a customised widefield fluorescence microscope and glycerol based mountant prior to imaging in a TEM (Johnson et al., 2015), or using STORM with a reducing buffer prior to imaging in a SEM (Kim et al., 2015). Here, we show that FPs also blink in dry mounted IRF sections, enabling *in vacuo* single molecule localisation and high-precision correlation of FP-labelled macromolecules to cell ultrastructure.

We illustrate the potential of this new high-precision correlative technique using HeLa cells infected with YFP-A3 Vaccinia virus (Arakawa et al., 2007) and HeLa cells transfected with GFP-C1. Viral particles are easily recognised based on morphology in electron micrographs; early immature virions (IV) are spherical and typically 280 nm in diameter, while mature virions (IMV, IEV) develop into brick shapes of 360 nm in the largest dimension (Condit et al., 2006; Cyrklaff et al., 2005; Sodeik and Krijnse-Locker, 2002). When labelled with YFP-A3, a core viral protein (Wilton et al., 1995), the fluorescent signal is punctate (Arakawa et al., 2007; Horsington et al., 2012). Viral particles in electron micrographs can therefore be used to assess the precision with which FPs can be located to the underlying structure (Kukulski et al., 2011). GFP-C1, which targets the lipid DAG at the endoplasmic reticulum, nuclear envelope, nucleoplasmic reticulum, and Golgi apparatus (Domart et al., 2012; Peddie et al., 2014), demonstrates that another FP from the same family in a different subcellular environment blinks under the same conditions, paving the way for multi-colour super-resolution CLEM.

2. Results

2.1. GFP and YFP blink in fixed whole cells at atmospheric pressure

To provide a baseline readout of FP blinking, we first imaged YFP-A3 vaccinia and GFP-C1 in whole fully-hydrated HeLa cells that were fixed in 4% PFA and mounted in Citifluor AF4 using the SECOM light microscope at atmospheric pressure in WF mode (Fig. 1). Both YFP and GFP displayed blinking behaviour when the laser power was increased to a density of 330 W/cm^2, and progressive photobleaching was apparent in the samples over the course of an image acquisition series of 4,000 frames (Fig. 1, supplementary movies 1 and 2). However, a substantial proportion of blinking molecules were obscured by out of focus fluorescence, which may lead to localisation errors. Super-resolution (SR) image reconstruction was carried out using ThunderSTORM (Ovesny et al., 2014). YFP-A3 localisation was punctate, as expected for individual virus particles (Fig. 1, upper panel). GFP-C1 was observed at the nuclear envelope as previously reported for DAG localisation (Fig. 1, lower panel), and in other cytosolic locations that could not be assigned to specific cellular organelles by fluorescence microscopy alone.

2.2. Workflow for SR CLEM in vacuo in the ILSEM

HeLa cells infected with YFP-A3 vaccinia or transfected with GFP-C1 were high pressure frozen, freeze substituted and embedded in HM20 resin as previously described (Peddie et al., 2014) (Fig. 2, step 1). Thin sections of ~ 200 nm were cut from the resin block (Fig. 2, step 2). Fluorescent cells were located using the SECOM light microscope in WF mode within the SEM at 200 Pa partial pressure of water vapour, which we previously demonstrated to be optimal for WF imaging *in vacuo* (Brama et al., 2015). The laser power was increased to a density of 330 W/cm^2, to drive FP blinking, and sequences of ~30,000 images were collected. The vacuum pressure was then decreased to 10^{-3} Pa (high vacuum) for optimal backscattered electron imaging. This WF-SR-EM cycle could be repeated sequentially to image different cells in the same section, or the same cell across serial sections to build up a 3D volume (Fig. 2, step 3). The SR images were reconstructed using ThunderSTORM to generate individual localisations (Fig. 2, step 4). Lastly, overlays of WF-EM and SR-EM were created to identify fluorescently labelled cellular structures (Fig. 2, step 5).

2.3. YFP-A3 blinks in vacuo, enabling SR CLEM localisation of YFP-A3 to the core of vaccinia virus

Reconstructed SR images of YFP-A3 vaccinia-infected HeLa cells in IRF sections *in vacuo* were of higher resolution than WF images of the same section (Fig. 3a; SMovie 3). Resolution was measured using Fourier Ring Correlation (FRC) (Nieuwenhuizen et al., 2013) to be 85.5 ± 13.1 nm, assessed using 10 patches of size 2.7 μm^2 across the image and twenty statistical repeats. Only patches that contained a significant amount of data were used in the evaluation since there were substantial areas of the image with very low information content. While this resolution was slightly lower than that typically achieved using organic dyes (e.g. 60 nm for tubulin labelled with Alexa 647 in Nieuwenhuizen et al. (2013)), this was unsurprising as the photon yields of FPs are typically lower. After WF and SR image acquisition, EM images were acquired in high vacuum (Fig. 3b). Composite images were generated to demonstrate the precision of correlation between WF-EM and SR-EM (Fig. 3c). The punctate distribution of YFP-A3 and the recognisable ultrastructure of the virus particles aided the alignment of light and electron images. YFP-A3 localised to viral particles from a range of developmental stages (Fig. 3c and d). Though the lateral resolution of the WF image was sufficient to correlate fluorescence to individual dispersed viral particles, it was difficult to resolve individual viral particles within clusters (Fig. 3c). In contrast, the lateral resolution of the SR reconstruction allowed correlation of fluorescent signal to the cores of individual virus particles (Fig. 3d). The precision with which the YFP signal could be localised to virus particle structure in WF-EM overlays compared with SR-EM overlays was assessed using a standard 2-colour Pearson's correlation coefficient measurement; a more negative coefficient reflecting a stronger correlation, since the viral particles were darker in the SEM image, and brightness was therefore anti-correlated. The WF-EM correlation was found to be −0.091, −0.002 and −0.053 (Fig. 3c, left to right, respectively), whereas the SR-EM correlation was considerably higher, at −0.165, −0.135, and -0.147 (O) (Fig. 3d, left to right, respectively), confirming the visual observation that in these images the SR signal was more strongly localised to viral particles.

2.4. GFP-C1 blinks in vacuo, enabling SR CLEM localisation of the DAG in cell membranes

Since GFP has previously been shown to blink on a longer timescale than YFP (Bagshaw and Cherny, 2006; Dickson et al., 1997),

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