Journal of Structural Biology 186 (2014) 205–213

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

Correlative Light- and Electron Microscopy with chemical tags

Mario Perkovic [a], Michael Kunz [a], Ulrike Endesfelder [b], Stefanie Bunse [c], Christoph Wigge [a], Zhou Yu [a], Victor-Valentin Hodirnau [a], Margot P. Scheffer [a], Anja Seybert [a], Sebastian Malkusch [b], Erin M. Schuman [c], Mike Heilemann [b], Achilleas S. Frangakis [a,*]

[a] Goethe University Frankfurt, Buchmann Institute for Molecular Life Sciences and Institute for Biophysics, Max-von-Laue Str. 15, 60438 Frankfurt am Main, Germany
[b] Goethe University Frankfurt, Institute for Physical and Theoretical Chemistry, Max-von-Laue Str. 13, 60438 Frankfurt am Main, Germany
[c] Max-Planck-Institute for Brain Research, Max-von-Laue Str. 4, 60438 Frankfurt am Main, Germany

ARTICLE INFO

Article history:
Received 19 February 2014
Received in revised form 20 March 2014
Accepted 24 March 2014
Available online 31 March 2014

Keywords:
Correlative electron and light microscopy
Electron tomography
Adherens junctions

ABSTRACT

Correlative microscopy incorporates the specificity of fluorescent protein labeling into high-resolution electron micrographs. Several approaches exist for correlative microscopy, most of which have used the green fluorescent protein (GFP) as the label for light microscopy. Here we use chemical tagging and synthetic fluorophores instead, in order to achieve protein-specific labeling, and to perform multi-color imaging. We show that synthetic fluorophores preserve their post-embedding fluorescence in the presence of uranyl acetate. Post-embedding fluorescence is of such quality that the specimen can be prepared with identical protocols for scanning electron microscopy (SEM) and transmission electron microscopy (TEM); this is particularly valuable when singular or otherwise difficult samples are examined. We show that synthetic fluorophores give bright, well-resolved signals in super-resolution light microscopy, enabling us to superimpose light microscopic images with a precision of up to 25 nm in the *x*–*y* plane on electron micrographs. To exemplify the preservation quality of our new method we visualize the molecular arrangement of cadherins in adherens junctions of mouse epithelial cells.

1. Introduction

Correlative Light- and Electron Microscopy (CLEM) is an approach that complements the information from two microscopic contrasts. Imaging of fluorescently labeled proteins localized by light microscopy (LM) is combined with the visualization of subcellular structures by electron microscopy (EM). To date, super-resolution light microscopy techniques can achieve a protein localization in the range of ~20 nm, whereas biological EM of thin-sectioned, resin-embedded samples yields a resolution of ~2–6 nm. With CLEM, superimposed images allow the protein of interest to be localized in its cellular context within a few tens of nanometers, on specimen preserved at close-to-native conditions. If optimized CLEM can yield images with such an unprecedented richness of information that is expected to surpass traditional immuno-gold labeling techniques in terms of precision, sample preservation, versatility and interpretation capabilities.

CLEM can be applied as a pre- or post-embedding technique, depending on whether the LM is performed before or after the embedding for EM. Technically, when LM is performed prior to fixation, there is a potentially small time delay (Verkade, 2008), in which the specimen can still change. Furthermore, samples might change during the EM embedding procedure, which might also obstruct CLEM. In general, the correlation of LM with EM data is challenging, since the *z*-resolution of the LM is much worse than the required thickness for TEM. Thus CLEM is preferably performed directly on sections on the TEM grid. For the imaging of thick samples without section preparation, block-face scanning electron microscopy can be performed (Denk and Horstmann, 2004; Heymann et al., 2006; Knott et al., 2008). This allows the generation of large volume data sets for 3D analysis of the sample in an automated fashion as well as CLEM (Murphy et al., 2011; Narayan et al., 2013).

Traditionally, protein-localization in TEM is performed by immuno-gold labeling. The gold could also be replaced by fluorophores for super-resolution microscopy. Both methods depend strongly on the quality of the available antibodies and accessibility of the epitopes on the surface (Nanguneri et al., 2012). Most closely related to the immuno-based techniques are quantum dots, which are both fluorescent and have a dense metal core that can be visualized in the EM. For increased specificity, protocols employing genetically-encoded tags applicable to photo-oxidation that utilize Tetracysteine/ReAsH (Gaietta et al., 2002), GFP (Grabenbauer et al.,

* Corresponding author.
E-mail address: achilleas.frangakis@biophysik.org (A.S. Frangakis).

http://dx.doi.org/10.1016/j.jsb.2014.03.018
1047-8477/

2005), and miniSOG/FMN (Shu et al., 2011) have been developed, these tags are suitable for photo-oxidation yielding an enrichment of electron-dense osmium tetroxide after illumination (reviewed in Ellisman et al. (2012)).

Electron microscopy of vitrified samples offers conditions closest to the native state (Sartori et al., 2007; Schwartz et al., 2007). However, vitrified samples can only be imaged at liquid nitrogen temperatures, which puts serious constraints on the light microscopy, prohibiting the use of super-resolution techniques as well as the use of high numerical aperture objectives, thus limiting the localization precision to a few hundred nanometers (van Driel et al., 2009). Notably, cryo-fixation techniques are generally considered to be the best methods for preserving biological samples in their native state and high-pressure freezing (HPF) is often implemented as a method in CLEM (Muller-Reichert et al., 2007). Most recently, HPF and freeze substitution (FS) EM-preparation protocols have managed to preserve the post-embedding fluorescence of GFP and other fluorescent proteins (Kukulski et al., 2011; Watanabe et al., 2011). Thereby, excellent protein localization has been achieved either by employing fiducials or by using super-resolution LM techniques. While these post-embedding fluorescence-retaining methods currently represent the state-of-the-art in CLEM, they have a particular disadvantage. These approaches require very low concentrations of heavy metals during sample preparation to avoid quenching of GFP fluorescence. However, heavy metals (uranyl acetate in particular) are important during the embedding procedure for structure preservation in EM. Thus, to date, a compromise between localization precision, fluorescence preservation, and EM quality has had to be made.

Here we use genetically-encoded chemical tags to label different cellular proteins with synthetic fluorophores, in order to circumvent the fluorescence quenching of GFP. We use SNAP (Keppler et al., 2003) and Halo (Los et al., 2008) tags expressed in living cells, that are coupled with the synthetic fluorophores Alexa Fluor 647, tetramethylrhodamine (TMR) and silicon-containing rhodamine derivative SiR-carboxyl (SiR). We show that these synthetic fluorophores conserve their post-embedding fluorescence independently of the uranyl acetate (UA) concentration, thus allowing CLEM for HPF/FS EM-preparation protocols. We demonstrate that our CLEM approach is suitable for simultaneously identifying the localization of up to two intracellular proteins using conventional fluorescence microscopy (FM) correlated with both TEM and SEM, thus opening up a new range of applications. Furthermore, it is easily possible to localize intra- and extracellular proteins of interest, with a few tens of nanometers precision, by combining super-resolution light microscopy and TEM.

2. Results

2.1. Preparation

CLEM allows the functional assignment of proteins prior to their EM analysis. Here we use a pre- and post-embedding CLEM approach. During HPF and FS we varied the concentrations of UA. We found that the post-embedding fluorescence of the synthetic fluorophores was well preserved at all UA concentrations (Fig. 1a–d). This is in contrast to GFP-based fluorescence retaining methods (Kukulski et al., 2011; Watanabe et al., 2011), which either avoid the use of UA, or keep it at an extremely low concentration in order to avoid fluorescence quenching. We find that 2% UA provides the best subcellular structural preservation, which is consistent with a previous report (Hawes et al., 2007). UA concentrations even higher than 2% might be advantageous for SEM imaging, since the higher heavy metal concentration increases the amount of detectable backscattered electrons. We quantified the quenching of the fluorescence of embedded L-cells stably expressing SNAPf-NCadherin labeled with Alexa Fluor 647. We measured the intensity of several individual cell contours in live-cell confocal imaging and compared the fluorescence signal after embedding using exactly the same optical settings. The specific fluorescence in the embedded samples is decreased to ~25%, and is not decreased further at higher UA concentrations. We find that the auto-fluorescence increases in the green channel only in the area of the embedded cells by ~7-fold (Fig. 1e and Supplementary Fig. 2). Interestingly this was consistent on all investigated specimen, thus we further explored this for automated image superposition of post-embedding LM images and EM micrographs.

2.2. Computational image superposition

The precision of superposition of the FM images on the EM micrographs is important for accurately localizing the labeled proteins within the EM micrographs. Both for embedded single cells and for cell layers the cell boundaries were well resolved by their auto-fluorescence (Fig. 1c, d and Supplementary Fig. 2) providing sufficient signal for positional identification, when compared to the cell membranes visible in the EM micrographs (Supplementary Fig. 1). The affine transformations were necessary to compensate for distortions due to the different orientation of the sample in the optical path as well as sample shrinkage due to electron irradiation.

2.3. SEM experiments

We visualized the embedded L-cells stably expressing SNAPf-NCadherin labeled with Alexa Fluor 647 using confocal fluorescence microscopy (Fig. 2a); the sample was subsequently trimmed to a block face for ion-abrasion SEM. Then, we selected a region containing cells with several cell–cell junctions for ion abrasion SEM (Fig. 2a box). In the SEM, we used backscattered electrons to record an image stack of $33 \times 28 \times 17\ \mu m^3$ with a pixel size of ~8 nm perpendicular to the beam direction, and ~20 nm along the beam direction. The computational slices from the image stack show a good structural preservation with sufficient contrast to recognize different cellular organelles (Fig. 2c). The N-cadherin fluorescence signal (red) overlapped with the positions of the junctions seen in the SEM, and in certain locations with the positions of filopodia on the cell surface, which should contain cadherins (Fig. 2c and d).

2.4. CLEM with various fluorophores

The wide range of genetically encoded chemical tags in combination with an even larger range of synthetic fluorophores offers various options for CLEM. Here we tested several tag-fluorophore combinations. In the first experiment we used HeLa cells stably expressing SNAPf-Histone 2B, which were labeled with TMR to show the feasibility of intra-nuclear labeling (Fig. 3a–d). In the overlay images of labeled Histone 2B (Fig. 3c), we observed that the fluorescence (orange) is excluded from the electron dense nucleolus that is separated from the electron lucent area of the nucleus showing areas of chromatin, in which the Histone 2B fluorescence signal is seen. To further verify the Histone 2B localization we recorded intranuclear tomograms (Fig. 3c box). In the tomographic slices the individual nucleosomes cannot be discerned, however the individual nuclear compartments can be clearly seen, and FM images superimpose nicely (Fig. 3d). In the second experiment we used HeLa cells stably expressing SNAPf-Cytochrome c oxidase subunit 8A labeled with TMR (Fig. 3e–h) to demonstrate cytoplasmic labeling. We find that the fluorescence of mitochondrial protein Cytochrome c oxidase (orange) is localized inside