Ultramicroscopy 143 (2014) 33–40

Contents lists available at ScienceDirect

Ultramicroscopy

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

Correlative cryogenic tomography of cells using light and soft x-rays

Elizabeth A. Smith [a,c], Bertrand P. Cinquin [a,c], Myan Do [a,c], Gerry McDermott [a,c], Mark A. Le Gros [a,b,c,*], Carolyn A. Larabell [a,b,c,*]

[a] Department of Anatomy, School of Medicine, University of California San Francisco, San Francisco, CA, United States
[b] Physical BioSciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA, United States
[c] National Center for X-ray Tomography, Advanced Light Source, Berkeley, CA, United States

ARTICLE INFO

Available online 7 November 2013

Keywords:
Cell structure
Imaging
Molecular localization

ABSTRACT

Correlated imaging is the process of imaging a specimen with two complementary modalities, and then combining the two data sets to create a highly informative, composite view. A recent implementation of this concept has been the combination of soft x-ray tomography (SXT) with fluorescence cryogenic microscopy (FCM). SXT–FCM is used to visualize cells that are held in a near-native, cryopreserved. The resultant images are, therefore, highly representative of both the cellular architecture and molecular organization in vivo. SXT quantitatively visualizes the cell and sub-cellular structures; FCM images the spatial distribution of fluorescently labeled molecules. Here, we review the characteristics of SXT–FCM, and briefly discuss how this method compares with existing correlative imaging techniques. We also describe how the incorporation of a cryo-rotation stage into a cryogenic fluorescence microscope allows acquisition of fluorescence cryogenic tomography (FCT) data. FCT is optimally suited for correlation with SXT, since both techniques image the specimen in 3-D, potentially with similar, isotropic spatial resolution.

1. Introduction

Microscopes for biological research come in all shapes and sizes, and range in complexity from simple light microscopes to highly sophisticated instruments that push the limits of existing technologies. Individually, all of these microscopes have one thing in common; they can only image specific cellular characteristics, and cannot provide us with a highly complete view of both cell ultrastructure and molecular organization. As a result, it is rare that data from any one modality can answer the types of questions posed in cell biology. To overcome this shortfall much effort has been directed towards the development of correlative microscopy, where the specimen is imaged by two complementary modalities and the data combined to generate a significantly more comprehensive view of the specimen. In the case of correlated light- and electron microscopy (CLEM) this approach has proven to be very effective [1–10]. CLEM is a highly informative combination of modalities, and remains the focus of significant on-going development. That said, there is still a pressing need to develop new correlative techniques, in particular using techniques that combine modalities with different image contrast mechanisms to electron microscopy [11–18]. Here we outline recent progress in combining soft x-ray tomography (SXT) with high-numerical aperture, fluorescence cryogenic microscopy (FCM) to create a new correlative method. SXT is used to quantitatively image cells and their sub-cellular structures [11,19]. FCM is used to generate molecular localization data, and/or confirm the identity of structures and organelles in SXT reconstructions [11,20–23]. The combination of these two modalities permits an enormous array of cellular features to be identified and quantified. In short, the output of correlated SXT–FCM is significantly more than the sum of the component modalities. Below we will first briefly describe alternative correlative microscopies to SXT–FCM, in particular CLEM, to highlight the unique niche filled by this new correlated modality. We will then describe the important characteristics of both SXT and FCM, particularly those characteristics that make them well suited to correlated studies. Finally, since correlated SXT–FCM is a relatively new technique, we will close by discussing the near term future prospects, the most exciting of which is equipping the FCM with a rotation stage that enables acquisition of fluorescence cryogenic tomographic (FCT) data. The application of tomographic methods to FCM greatly reduces anisotropy in the fluorescence signal (in standard light microscopy approaches the spatial resolution is significantly better in x and y than it is in z, i.e. the axis along the illumination light path through the specimen) and allows molecules to be localized with much greater precision. Since FCT and SXT are both 3-D techniques, and potentially produce data with similar spatial accuracy and precision, these two types of reconstruction can be confidently correlated.

As with the individual modalities, the combination of SXT–FCT can be applied to a wide range of cell types, ranging from small bacteria, through to large eukaryotic cells. Any cell that falls within

* Corresponding authors at: Department of Anatomy, University of California San Francisco, 1550 4th Street, Box 2722, San Francisco, CA 94143-2722, United States. Tel.: +1 415 514 0423.
E-mail addresses: MALegros@lbl.gov (M.A. Le Gros), carolyn.larabell@ucsf.edu, calarabell@lbl.gov (C.A. Larabell).

0304-3991/$ - see front matter © 2013 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ultramic.2013.10.013

the size constraints placed by SXT is suitable (i.e. a maximum thickness of 15 μm). As will be described below the specimen mounting system used determines the overall characteristics of the specimens that can be imaged [17]. Large adherent cells are imaged on TEM style grids [14]. Cells grown in suspension – provided they meet the size criteria above – are best imaged when mounted in thin-walled glass capillary tubes [24]. The fluorescent signal can be derived from any of the labeling methods commonly used in cell biology (genetically encoded fluorescent proteins or small molecule stains). Consequently, SXT–FCT can meet most imaging needs in cell biology, whether it is imaging the effect of candidate drug molecules on pathogenic microbes, or determining the structural consequence of genetic mutations or environmental factors on human cells.

2. Comparison of SXT–FCM with other correlative techniques

In CLEM the cell ultrastructure is visualized by electron microscopy and molecular localization using fluorescence. Of course, molecules can also be localized directly in an in electron micrograph if they have been labeled with electron-dense tags. However, given the enormous body of cell biology research that has been carried out using FP labeled molecules [25–27] it is highly advantageous to combine ultrastructural imaging with fluorescence data [28]. Adopting this approach has presented a number of challenges, many of which have been successfully overcome in cases where the specimen has been chemically fixed [10]. However, in cryo-EM obtaining fluorescence data from a cryopreserved specimen has proven more difficult. To date, the fluorescence microscopes used in cryo-CLEM studies have all employed low numerical aperture (NA) air lenses (that is to say, an NA of 1.0 or lower) [7–9,29–31]. Lenses such as these are not well matched to the refractive index (RI) of the specimen. The mismatch in the RI of air compared to the specimen leads to blurring and a reduction in the precision with which molecules can be localized [20]. The major advantage of FCM is the use of lenses that are coupled to the specimen by an index-matched immersion fluid (such as propane, RI 1.32). This coupling greatly improves image quality, and allows the use of higher numerical aperture lenses [20,21,23].

3. Specimen mounting and preservation for correlative SXT and FCM

As with any correlated imaging experiment, the SXT–FCM specimen mounting system must be robust, easy to handle, and not interfere with image acquisition in either microscope. Currently, two different systems predominate; one based on TEM style grids [13,32,33], the other, thin-walled capillary tubes [17,24,34,35]. Naturally, each has inherent advantages and disadvantages. TEM grids can be used to mount large, adherent cells, and even allow cells to be cultured in situ on the grid. Whilst this is a very positive advantage it also places restrictions on the rotation of the specimen to a maximum of $\pm 70^{\circ}$ [32]. As the grid is tilted the specimen becomes thicker with respect to the illumination axis, and therefore more strongly absorbing, eventually reaching a point where there is insufficient transmission of the specimen illumination [32]. Systematically missing data associated with limited tilt tomography negatively impacts tomographic reconstructions and leads to obvious artifacts. On the other hand, when the specimen is mounted in a thin-walled glass capillary it can be viewed from any perspective without an apparent increase in thickness. The disadvantage, however, is that the capillary diameter is restricted to ~ 15 μm (i.e. the maximum thickness of specimen that can be imaged by SXT). In summary, the decision between which specimen mounting system to use comes down to the size of specimen being imaged; for cells up to 15 μm in size capillaries are the optimal choice (for completeness of data), for large, extended specimens a TEM grid is the only choice if the specimen is to be imaged intact (capillaries can be used to mount large cells if they are resuspended, or even tissue sections if they are sectioned using a microtome, or de-bulked using techniques such as ion milling). The correlated imaging system developed at the National Center for X-ray Tomography (ncxt.lbl.gov) has focused primarily on the use of glass capillaries. The walls of these capillaries are 250–400 nm thick; this is thin enough that both x-ray and light microscopy can be performed with minimal degradation in the signal [36]. When mounted in a suitable rotation stage, a capillary can be rotated to any arbitrary angle for tomographic data acquisition [22–24]. The importance of this factor cannot be understated when the goal is collection of tomographic data since the reconstruction algorithms are highly sensitive to missing data (as occurs when rotation of the specimen is limited) [37].

In all tomographic methods the specimen is imaged a number of times [38]. Repeated exposure of the specimen to harsh illumination has, of course, the potential to cause damage, particularly in x-ray imaging where damage is cumulative as a function of received dose [39–41]. Preserving the specimen, either chemically or cryogenically, can mitigate damage during data acquisition, at least to the point it is not visible in the image [11,36]. Cryopreservation is generally accepted to be the preferred method, since this has been shown to retain the fine structural details and in vivo molecular organization much more effectively than chemical fixation [43–45]. Consequently, cryopreservation is the preferred fixation method for correlated SXT–FCM [23,32]. In addition, cryopreservation has the added benefit of increasing the working lifetime of the fluorescence labels, typically by a factor of 30 or greater [20,46]. This increase in working lifetime makes fluorescence tomography viable, since the fluorescence signal remains largely constant throughout the process of image acquisition. Again, this is an enormously significant factor, since loss of fluorescence in the later stages of data collection would impart significant noise into the tomographic reconstruction.

4. High numerical aperture fluorescence cryo-microscopy

Fluorescence microscopy (FM) is one of the most commonly performed imaging techniques in cell biology, and as such needs little further discussion beyond acknowledging the power of genetically encoded probes, and the ability to label almost any molecule in a cell with a fluorescent tag of a chosen color [26]. This technology has been revolutionary, and has made an enormous impact in the scientific literature. Therefore, FM is an obvious partner to use in conjunction with a modality that visualizes cell structure.

Early work aimed at correlating SXT and FM data relied on room-temperature confocal fluorescence microscopy of chemically fixed, dehydrated specimens [47]. Whilst interesting and significant in the field, this work fell short of the mark in terms of being informative. For example, the cells were undoubtedly far from representative of their in vivo state by virtue of the fixation method used and the fact that the cells were dehydrated. However, this work made the goal for future developments patently clear – to create a system that allows specimens to be imaged in a near-native state, and with fluorescence imaging that has the best possible fidelity. This meant collecting FM data from cryo-preserved, rather than chemically fixed, dehydrated specimens, and using cryogenic immersion fluid, rather than air, to couple the lens to the specimen [22]. Since no commercial microscopes were available that met these criteria, NCXT staff built a custom