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Correlative microscopy methods that maximize specimen fidelity and data completeness, and improve molecular localization capabilities

Elizabeth A. Smith [a], Bertrand P. Cinquin [a], Gerry McDermott [a], Mark A. Le Gros [a,b], Dilworth Y. Parkinson [c], Hong Tae Kim [d], Carolyn A. Larabell [a,b,*]

[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] Advanced Light Source, Lawrence Berkeley National Laboratory, Berkeley, CA, United States
[d] Department of Anatomy, School of Medicine, Catholic University of Daegu, South Korea

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ABSTRACT

Correlative microscopy techniques interrogate biological systems more thoroughly than is possible using a single modality. This is particularly true if disparate data types can be acquired from the same specimen. Recently, there has been significant progress towards combining the structural information obtained from soft X-ray tomography (SXT) with molecular localization data. Here we will compare methods for determining the position of molecules in a cell viewed by SXT, including direct visualization using electron dense labels, and by indirect methods, such as fluorescence microscopy and high numerical aperture cryo-light microscopy. We will also discuss available options for preserving the *in vivo* structure and organization of the specimen during multi-modal data collection, and how some simple specimen mounting concepts can ensure maximal data completeness in correlative imaging experiments.

1. Introduction

Imaging is a key technique in cell biology, and the origin of much of our understanding of cell structure and organization (Leis et al., 2009). Cellular imaging is typically carried out to either visualize and quantify sub-cellular structures, or measure the cellular distribution of specific molecules (Sartori et al., 2005). Normally both types of information are needed to fully answer a biological question, irrespective of the question being asked or the type of cells being imaged (Leis et al., 2009). Clearly, the optimal situation is to collect *both* types of data from the *same* specimen because this imparts confidence in the validity of any conclusions drawn compared with the alternative of making assumptions based on data acquired from different specimens (Giepmans et al., 2005; Le Gros et al., 2009; Lucic et al., 2007; Martone et al., 2000; Sartori et al., 2007). As a result there has been an enormous upswing in the development and use of so-called 'correlated microscopies'.

In correlated microscopies a specimen is imaged using two or more microscopes and the data is combined to form a composite view. Whilst this approach to imaging cells is highly desirable, the methodology required poses a number of technical and instrumental challenges, which until recently proved daunting and difficult to overcome (Leis et al., 2009, 2006; Sartori et al., 2005). Firstly, the specimen must remain loyal to the *in vivo* state for the duration of data collection, both in terms of the cell's structure and organization. Secondly, data acquisition by one modality must not compromise either the fidelity of the specimen or the ability to carry out subsequent imaging methods. Thirdly, the data obtained from all modalities should be as complete as possible since missing data can mask or skew important features in the specimen, resulting in errors in assignment of location, quantification, or in determining the presence of absence of particular molecules. Here, we will discuss methods that have been developed for correlating soft X-ray tomography (SXT) with molecular localization methods, with a particular emphasis on fluorescence microscopy (FM).

Since SXT may not yet be familiar to all readers we will now briefly describe the characteristics and attributes of this modality as stand-alone techniques, prior to describing how it can be combined and correlated with molecular localization methods.

2. Soft X-ray tomography

Soft X-ray microscopes currently used for studying biological material measure the transmission of "soft" X-ray photons through a specimen (Attwood, 1999). "Soft" X-ray photons have energies

Abbreviations: CFM, cryogenic fluorescence microscopy; FM, fluorescence microscopy; LAC, linear absorption coefficient; PSF, point spread function; SXT, soft X-ray tomography; YFP, yellow fluorescent protein.

* Corresponding author. Address: Department of Anatomy, University of California San Francisco, 513 Parnassus Avenue, Box 0452, San Francisco, CA 94143-0452, United States.
E-mail addresses: calarabell@lbl.gov, carolyn.larabell@ucsf.edu (C.A. Larabell).

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that fall within the so-called 'water window' region of the spectrum (Kirz et al., 1995). That is to say, between the K-absorption edges of oxygen at 280 eV and carbon at 530 eV (this equates to 2.34 and 4.4 nm respectively) (McDermott et al., 2012b). At these energies, the illuminating light is attenuated an order of magnitude more strongly by biological materials than by water (Attwood, 1999; Kirz et al., 1995; Larabell and Le Gros, 2004a; Larabell and Nugent, 2010; Schneider, 1999, 2003; Schneider et al., 2001; Schneider et al., 2003). This difference is linear, adheres to the Beer–Lambert Law and – because biological specimens are highly varied in terms of their internal composition – gives rise to excellent image contrast in most specimens, particularly biological cells (Attwood, 1999; Kirz et al., 1995; Larabell and Le Gros, 2004a; Larabell and Nugent, 2010; Schneider, 1999, 2003; Schneider et al., 2001; Schneider et al., 2003).

Soft X-ray microscopes make use of Fresnel zone plate condenser and objective lenses that have low numerical aperture and relatively large depth of focus (Attwood, 1999; Kirz et al., 1995; Larabell and Le Gros, 2004a; Larabell and Nugent, 2010; Schneider, 1999, 2003; Schneider et al., 2001; Schneider et al., 2003). Therefore, images taken using the X-ray microscope of specimens that are on the order of 10 μm in diameter are assumed to be 2-dimensional projections of the transmission through the specimen (Larabell and Le Gros, 2004a). Soft X-ray microscopy is combined with tomography, which involves simply imaging the specimen from a number of different angular viewpoints (Larabell and Le Gros, 2004a). If a sufficient number of 2-dimensional images are collected, a 3-dimensional reconstruction of the specimen can be calculated (Weiss et al., 2000).

The fluence of X-ray photons required for soft X-ray tomography could cause serious structural damage to a biological specimen. Damage is generally cumulative with dose, and therefore a serious concern in techniques when using tomography because the specimen is repeatedly illuminated (Fischer et al., 2006; Weiss et al., 2000). The long-standing solution to this problem has been to 'preserve' or 'fix' the specimen, either chemically using cross-linking aldehydes or by quickly cooling the specimen to low temperature (usually liquid nitrogen temperature, or lower) (Baumeister, 2002; Baumeister et al., 2008; Cyrklaff et al., 2007; Leis et al., 2009, 2006; Nicastro et al., 2000). There is an additional advantage to preserving the specimen; this process locks structures and molecules in place and prevents them from moving during data acquisition (Leis et al., 2006; Quintana, 1994; Ryan, 1992). Again this is particularly important in techniques where multiple images, or imaging modalities are required.

As with any process carried out prior to imaging, it is enormously important that the fixation procedure retains the *in vivo* structure and organization of the specimen (McDermott et al., 2009). Otherwise the resulting images will not represent reality, and be a source of confusion. Chemical fixation is a well-established technique that has been very commonly used in fluorescence, electron and other microscopies (Bell and SafiejkoMroczka, 1997). There is a substantial body of evidence – initially from electron microscopy and subsequently by soft X-ray microscopy – showing that chemical fixation can devastate the structural integrity of a specimen, even when carried out carefully by an expert (Leis et al., 2009). As a result, cryopreservation via vitrification is now considered the vastly preferred of the two approaches for any high resolution imaging study (Al-Amoudi et al., 2004; Baumeister et al., 2011; Dubochet et al., 1981, 1988; Leforestier et al., 1996; Leis et al., 2006). There are other significant benefits to cryopreservation for biological imaging. Specimens can be conveniently fixed at discrete points in time allowing time-dependent experiments to be carried out easily. For example, populations of cells can be frozen after a change in their environment, initiation of an event sequence, such as the cell cycle, or the introduction of a candidate drug molecule into the growth media. Moreover, large numbers of specimens can be collected and preserved, and then imaged when convenient or practical. This makes acquisition of statistically significant numbers of cellular imaging data set feasible. Consequently, all soft X-ray microscopes used for biological imaging incorporate instrumentation to maintain the specimen at cryogenic temperatures. Prior to being mounted in the microscope, the specimen can either be rapidly plunged into liquid cryogen, or fixed in a high-pressure freezer (Baumeister et al., 2011).

After selecting the type of preservation, the next consideration is how to mount the specimen for SXT imaging. There is a wide-range of possible options; for example, thin silicon nitride windows, or the grid system commonly used the electron microscopy community. Thin-walled glass capillaries have been developed and adopted as the specimen mount at the National Center for X-ray Tomography soft X-ray microscope (Larabell and Nugent, 2010; McDermott et al., 2012a,b). Two very important factors contribute to making the capillary a good decision. Firstly, the geometry of a cylinder – mounted along the axis of rotation –means the specimen can be imaged from any arbitrary angle around a full 360° of rotation. This in turn allows projection series to be collected without the 'missing wedge' of data that is unavoidable if the specimen is mounted on a flat support (such as a silicon nitride window, or an electron microscopy grid) (Leis et al., 2009). Typically, such mounts can only be tilted a maximum of ±70°, and as the flat specimen is tilted it appears significantly thicker, and therefore more strongly absorbing, to the point that there is insufficient transmission of the specimen illumination to the detector to provide adequate signal-to-noise. Missing data and the other factors associated with limited tilt tomography negatively impact the resultant tomographic reconstructions (Hagen et al., 2012; Leis et al., 2009). No such problems are encountered if the specimen is mounted in a capillary. In this geometry the sample can be rotated completely and therefore the tomographic reconstructions are significantly freer from artifact (Le Gros et al., 2005; McDermott et al., 2012a,b).

As mentioned above, soft X-ray microscopes produce two-dimensional projection images of the specimen (Falcone et al., 2011; Larabell and Nugent, 2010). Fig. 1 shows a two-dimensional projection image of a human mammary epithelial cell taken using a soft X-ray microscope. The cell is obviously an internally complex three-dimensional object and as a consequence, such a projection image is virtually useless; all of the organelles and cellular structures appear confusingly superimposed on top of each other (Derosier and Klug, 1968). Real information about cellular structure requires three-dimensional information. Fortunately, calculation of a three-dimensional tomographic reconstruction of the cell from the two-dimensional projection images is a straightforward, commonly used, and a very well established technique (Natterer, 1986; Natterer and Wübbeling, 2001). For example, this is the principle behind medical computed tomography (CT) that is ubiquitous in hospitals and clinics throughout the world. The basic concepts behind CT are used in SXT, but at significantly higher resolution (better than 50 nm). In SXT, the X-ray source and detector remain fixed and the specimen is rotated inside the microscope (Larabell and Nugent, 2010).

Fig. 2F shows a computer-generated slice through the center of a soft X-ray reconstruction of a vitrified mouse lymphocyte. The spatial resolution of this soft X-ray reconstruction is ~50 nm, and the contrast reflects the distribution of water and organic matter inside the cell. It has been observed that in soft X-ray reconstructions of biological specimens the major organelle types display characteristic attenuation patterns (McDermott et al., 2012a). For example, lipid droplets appear dense (highly absorbing) due to the high density of biomolecules and low water content. Structures