Synchrotron-Based X-Ray-Sensitive Nanoprobes for Cellular Imaging

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It is one of the ultimate goals in cell biology to understand the complex spatio–temporal interplay of biomolecules in the cellular context. To this end, there have been great efforts on the development of various probes to detect and localize specific biomolecules in cells with a variety of microscopic imaging techniques. In this Research News, we first summarize several types of microscopy for visualizing specific biomolecular targets. Then we focus on recent advances in the design of X-ray sensitive nanoprobes for applications in synchrotron-based cellular imaging. With the availability of advanced synchrotron techniques, there has been rapid progress toward high-resolution and multi-color X-ray imaging in cells with various types of functional nanoprobes.

1. Introduction

In cell biology, we study cellular architecture and function to reveal underlying basic principles of life at the microscopic, submicroscopic and molecular levels. In this regard, microscopy plays a key role for studies in cell biology. Indeed, we have witnessed that every milestone progress in microscopy led to new insights and discoveries in cell biology. Dating back to the mid-1600s, the development of optical microscopes with microscale resolution by Leeuwenhoek made it possible to directly observe cells, which laid the foundation of cell biology. The sub-cellular structures and organelles, such as cell nucleus, mitochondria, Golgi apparatus and endoplasmic reticulum were imaged with the advent of electron microscopy. Since the last century, fluorescence microscopy provided a powerful tool to image cell with the ability of targeting specific biomolecules, which revolutionalized modern cell biology and remains a dominating tool in biological laboratories.[1] It is important to note that modern cell biologists are primarily interested in studying biomolecular locations and interactions under the cellular context, i.e. "functional" imaging (in contrast to the traditional "structural" imaging). The discovery of green fluorescent protein (GFP) and its further development is undoubtedly the major breakthrough for enabling functional imaging of the localization, transportation and distribution of important proteins inside the cells, as well as analysis of a variety of basic cellular processes such as transcription, translation, cell-cell signaling and apoptosis.

Despite its unparalleled abilities for cellular imaging, fluorescence microscopy is inherently limited by the optical resolution limit and spectral overlap within the visible light range. The appearance of super-resolution fluorescence microscopy circumvented the optical limit and achieved sub-100 nm imaging.[2] Synchrotron-based X-ray imaging offers an alternative approach to nanoscale cellular imaging.[3] These new methods hold great promise to improve our understanding of cellular structure and function, which might lead to new insights of cells at the unprecedented nanometer scale.

Synchrotron radiation provides significantly improved x-ray sources with the characteristics of high brilliance, high intensity, high level of polarization, high collimation, and wide tunability in energy/wavelength.[4] The synchrotron-based X-ray microscopy, with associated wavelength ranging from a few nanometers to even sub-nanometers, offers the potential to image objects at the nanoscale spatial resolution.[5,6] Also importantly, X-ray spectroscopy has excellent elemental specificity due to its extremely narrow spectral lines.[7,8] By exploiting X-ray absorption fine structure (XAFS) spectral features of specific elements, cellular localization of biomolecules could be achieved with functional probes with specific elements. Because of these unprecedented advantages, there have been great interests in design and synthesis of X-ray sensitive functional nanoprobes for X-ray imaging of cells. In this Research News, we will summarize recent advances in this direction and

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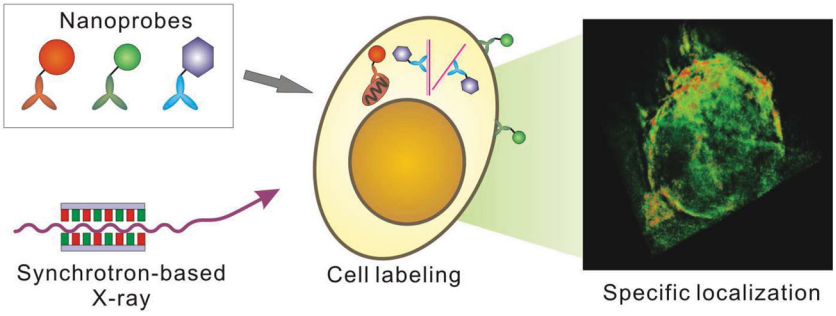

Scheme 1. Synchrotron-based nanoscale cellular imaging with X-ray sensitive nanoprobes.

show examples of promising applications in cellular imaging, with the particular attentions on nanometer-resolution and multi-color imaging (**Scheme 1**).

2. Brief Descriptions of Existing Microscopy for Cellular Imaging

2.1. Fluorescence Microscopy

Fluorescence has long been used to visualize cell biology at many levels. In general, fluorescence was observed from either small organic dyes or fluorescent proteins that are associated with a specific target protein via antibodies or co-expression. In fluorescence imaging, it is advantageous for studying a cellular process by labeling of several proteins. However, the large spectral overlap between fluorescent probes restricts its ability to simultaneously interrogate multiple targets. In a typical setting, fluorescence spectroscope can differentiate as much as four species of targeted proteins (**Figure 1**).[1] The optical resolution limit (~200–300 nm) forms the other major barricade for fluorescence microscopic imaging. This resolution is approximately the size of an intracellular organelle, hence it is highly demanding to develop new techniques to study the inner architecture of many subcellular structures as well as localization and imaging of intracellular proteins. During the past decade, the invention of super-resolution fluorescence microscopy provides a means to revolutionize the way to study cell biology by breaking the diffraction barrier and reaching the nanometer-scale resolution of imaging.[2] Stimulated emission depletion microscopy (STED) is the first type of super-resolution microscopy,[9] followed by saturated structured illumination microscopy (SSIM), stochastic optical reconstruction microscopy (STORM) and photoactivated localization microscopy (PALM).[10] Despite its major progress in resolution improvement, super-resolution fluorescence microscopy is largely hindered by its poor ability of simultaneous localization and mapping of multiple proteins due to the difficulty in coupling multiple dyes to the complex optical settings.

2.2. Raman Microscopy

Raman spectroscopy is a relatively newly developed, nondestructive imaging technique that allows multiple parameter analysis of live cells. Since Raman signals are intrinsically weak, the conventional Raman spectroscopy has limited sensitivity for cellular imaging.[11] To circumvent this drawback, metal nanoparticles (gold or silver) are introduced to induce the surface enhanced Raman scattering (SERS) that greatly improves the sensitivity of Raman. Metal nanoparticles are attached with reporter molecules (proteins or nucleic acids) to act as probes for targeted cellular imaging.[12] More recent studies showed that carbon nanotubes and graphene also exhibited intense Raman peaks that could be used as contrast agents for cellular imaging.[13] However, it should be noted that the excitation laser spot of the Raman microscope only can be focused at the micrometer scale, which offers limited spatial resolution for Raman-based cellular imaging.

2.3. Immunoelectron Microscopy

Immunoelectron microscopy relies on electron-dense ferritin or gold particle linked to specific antibodies for the labeling to determine the subcellular localization of specific proteins. Due to its ultrahigh resolution (<10 nm), immunoelectron microscopy has been popularly employed in cell biology for the studies on practically every unicellular and multicellular organism. Gold nanoparticles of different diameters enable two or more proteins to be simultaneously studied.[14] However, the sample preparation of immunoelectron microscopy is a complicated

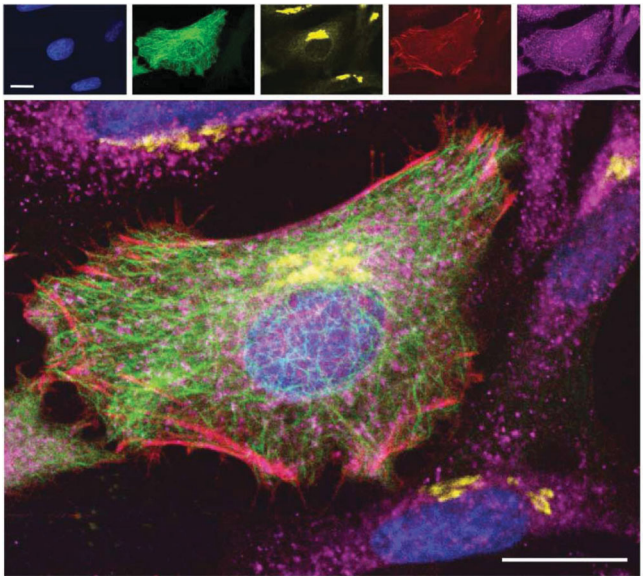

Figure 1. Parallel application of targeting methods and fluorophores. HeLa cells transfected with GFP–α-tubulin and tetracysteine–β-actin were stained with ReAsH. After fixation, cells were immunolabeled for the Golgi matrix protein giantin with QDs and for the mitochondrial enzyme cytochrome c with Cy5 as indicated. DNA was stained with Hoechst 33342. Individual channels are false-colored (middle) and merged (bottom). Scale bars, 20 μm.[1] Copyright 2006, American Association for the Advancement of Science.

and error-prone process, which includes fixation, dehydration, embedding, sectioning, immunostaining, and electron microscopy contrast staining.[15] In particular, contrast staining often brings about nano-sized contaminants that possibly result in artifacts.[16] More significantly, the indispensable slicing of cells results in mechanical distortions, which potentially means the loss of structural information in electron microscopy.[17] Newly developed environmental scanning electron microscopy (ESEM) dose not require any severe processing of the sample, lowering the risk of generating artifacts and interfere with the immune labeling procedure, and thus allows for imaging cell samples in, or close to, their native state.[18] However, there are also challenges in the use of ESEM such as fine features of cell samples are damaged by beam interactions.[19]

2.4. Atomic Force Microscopy

Atomic force microscopy (AFM) is a type of high-resolution scanning probe microscopy (SPM), with demonstrated resolution on the order of 1 nm.[20] AFM can not only be used to image cellular membranes and cytoskeletal structures, but also be used to analyze biological processes such as protein-protein interactions and assembly of membrane proteins.[21] However, simultaneous localization and mapping of multiple proteins by AFM is unachievable at present. Also, imaging artifacts remain a hurdle, which could arise from tip effects or sample preparation. More recently, there have been many research efforts on the coupling of AFM with total internal reflection fluorescence (TIRF) microscopes, which largely avoids these problems and provides a combination of morphological and functional information of the cells.

2.5. Synchrotron-based X-ray Microscopy

X-ray imaging is heavily dependent on synchrotron, and such devices have been built at synchrotrons over the world, including Advanced Light Source (ALS) at the Lawrence Berkeley National Laboratory in US, European Synchrotron Radiation Facility (ESRF) in France, BESSY-II in Germany, SPring8 in Japan, and three generations of synchrotrons in China (Beijing, Hefei and Shanghai), etc. X-ray microscopy employs X-ray wavelengths from 0.1–10 nm for imaging, which enables high-resolution imaging of tens of nanometers. This resolution can bridges the existing detection gap between light and electron microscopy. There are two major approaches to cellular imaging with X-rays from synchrotron sources – i) using zone plates, K-B optics, multilayer Laue lens, and compound refractive lens for focusing X-rays and ii) "lensless" approaches such as coherent diffractive X-ray imaging which utilize iterative algorithms to calculate phases from oversampled far field diffraction patterns.[22] Recent advances in focusing conditions have offered the potential of further increase of the spatial resolution of a few nanometers,[6] which holds the promise to observe single bio-macromolecules inside the cell.[23,24] In addition to its great imaging power, synchrotron-based X-ray microscopes provides a combination of techniques, e.g. absorption, phase, scattering and fluorescence, which can be used to observe and analyze a variety of physical changes and chemical reactions.[8,25] Compared with the electron beam, X-ray has a greater ability to penetrate biological specimens without the necessity of chemical fixation and sectioning of the specimen.[26]

There exists a "water window" in X-ray, which is the region of the spectrum that lies between the K shell absorption edges of carbon (284 eV, $\lambda = 4.4$ nm) and oxygen (543 eV, $\lambda = 2.3$ nm). In this range, X-rays are absorbed an order of magnitude more intensely by carbon- and nitrogen-containing organic material (proteins and lipids) than by water. Given this unique advantage, structures in the cell can be visualized directly as a function of both their density and biochemical composition under a high background of cellular water.[27] Therefore, X-ray microscopy offers the potential for imaging cells in a living, hydrated state, which could potentially show the natural, functional state of cells. For example, Larabell and coworkers obtained the first whole cell images of yeast, *Saccharomyces cerevisiae*, with X-ray tomographic reconstruction, which showed overviews of the subcellular structural organization including cell nucleus, vacuole and lipid-filled vesicles at 60-nm resolution.[28] More recently, Schneider et al. obtained three-dimensional reconstructions of 5-μm mouse adenocarcinoma cells with a resolution of 36 nm, which visualized the double nuclear membrane, nuclear pores, nuclear membrane channels, mitochondrial cristae and lysosomal inclusions (**Figure 2**).[29] In addition to the "water window", Nam et al. recently developed wet coherent x-ray diffraction microscopy for imaging fully hydrated and unstained biological specimens. Whole cell morphologies and internal structures with <25 nm resolution could be clearly visualized without contrast degradation.[30]

Coherent diffractive imaging (CDI) approaches have also begun to produce excellent images of biological samples at nanometer resolution.[31] For example, Nishino et al. observed the axial structure of unstained human chromosome with 38-nm 2D reconstruction resolution by using coherent X-ray diffraction.[32] Also Shapiro et al.[33] and Jiang et al.[34] have imaged organelles and other subcellular structures in whole cells using CDI. In the future, CDI of whole cells holds the potential for dynamic cellular imaging with X-ray free electron lasers (XFELs), although currently the technical challenges remain daunting, including sample destruction under the high-intensity laser, the role of nonlinearity, as well as computational reconstruction.[35]

Besides the topological imaging ability, X-ray spectroscopy provides excellent elemental specificity with narrow spectral lines. By exploiting the K- and M-edge X-ray absorption fine structure (XAFS) spectral features of a given element, cellular localization of proteins or nucleic acids can be imaged using functionalized nanoprobes with specific element labeling.[3] In addition, CDI approaches have also been used for element specific imaging.[36] By phasing coherent x-ray diffraction patterns below and above the absorption edge of a specific element, Miao and coworkers obtained cellular localization of functional probes with specific element labeled biomolecules.[22]

3. X-Ray-Sensitive Nanoprobe for Cellular Imaging

A variety of nanomaterials, especially quantum dots (QDs), have been employed as fluorescence probes to target specific

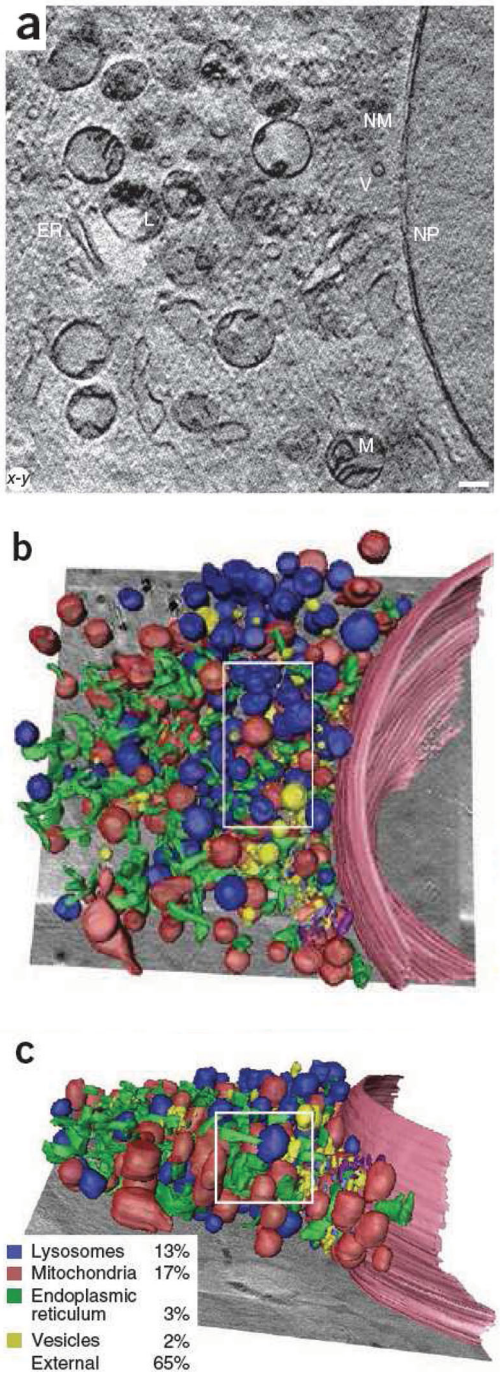

Figure 2. X-ray images of a cell. (a) The 3D partially coherent X-ray tomograms of mouse adenocarcinoma cells show many subcellular organelles including mitochondria (M), lysosomes (L), endoplasmic reticulum (ER), vesicles (V), the nuclear membrane (NM) and nuclear pores (NP). Scale bar, 0.39 μm. (b,c) The 3D data corresponding to the image in (a) were segmented, yielding *x-y* (b) and *x-z* (c) views of the cytoplasm. Percentages indicate the volume fraction occupied by different organelles measured in the 3D subvolume delineated by the white rectangles.[29] Copyright 2010, Nature Publishing Group.

biomolecules, and shown high promise in cellular imaging.[37] Given that many of these nanomaterials contain metal elements that have strong X-ray absorption, they can be explored as X-ray excitable nanoprobes to localize bio-macromolecules in cells with high resolution.[38] So far, several kinds of nanomaterials including gold nanoparticles (AuNPs), TiO_2 NPs and QDs have been employed for the development of X-ray sensitive nanoprobe for localizing specific biological targets. AuNPs and TiO_2 NPs are known to be of minimal cytotoxicity.[39,40] Although QDs are inherently cytotoxic, there have been elegant approaches to prepare core–shell–shell structured QDs that show low cytotoxicity.[41]

3.1. AuNPs

Au-labeled antibodies are popularly used for localizing proteins in immunoelectron microscopy. Larabell's group first demonstrated that the immunogold labeling protocol could be employed for protein localization with soft X-ray microscopy. The microtubule network in cells was labeled using primary antibodies, followed by incubation with 1.4-nm-AuNP-conjugated secondary antibodies. The bound AuNPs were then enhanced with Au seed-induced silver reduction to form aggregates of approximately 50 nm in diameter. This silver-enhanced AuNPs labeling proved to be an effective way to visualize the microtubule network in a whole, hydrated mouse epithelial cell (~8–10 μm thick) with a resolution of ~50 nm.[42] In a different study, by taking advantage of the characteristic X-ray fluorescence of Au, McRae et al. designed a dual label that combines an organic Xuorophore with a 1.4 nm AuNP as xenobiotic markers for synchrotron-based X-ray fluorescence (microXRF) microscopy. Mitochondria and the Golgi apparatus in mouse fibroblast cells were labeled with specific primary antibodies and then the dual-label nanoprobe. XRF images showed the location and structural details of the Au-labeled organelles in cells, which correlated well with the subcellular distribution visualized with optical fluorescence microscopy.[43]

3.2. TiO_2 NPs

TiO_2 NPs have been widely used in industries due to their photocatalytic activity and UV light absorption properties. Woloschak's group showed the potential of TiO_2–oligonucleotide nanocomposites as X-ray-sensitive probes for intracellular localization of oligonucleotide by using microXRF microscopy.[23] Ashcroft et al. also reported that TiO_2 nanoparticles could be used as effective probes for soft X-ray imaging. In their work, TiO_2 NPs were conjugated to streptavidin to form a nanoprobe, which were then incubated with biotinylated microtubules and examined using scanning transmission X-ray microscopy (STXM). The absorption image clearly showed that the dense filamentous microtubule was decorated with numerous NPs. The NPs were revealed in the titanium mapping while the carbon mapping image revealed microtubules. These data confirm the effectiveness of TiO_2–based nanoprobes for biolabeling.

Larabell and coworkers also showed that TiO_2 NPs could be combined with Au NPs to obtain high-resolution images about the location of two different proteins of interest by using soft X-ray microscopy.[44] In our research group, Cai et al. employed

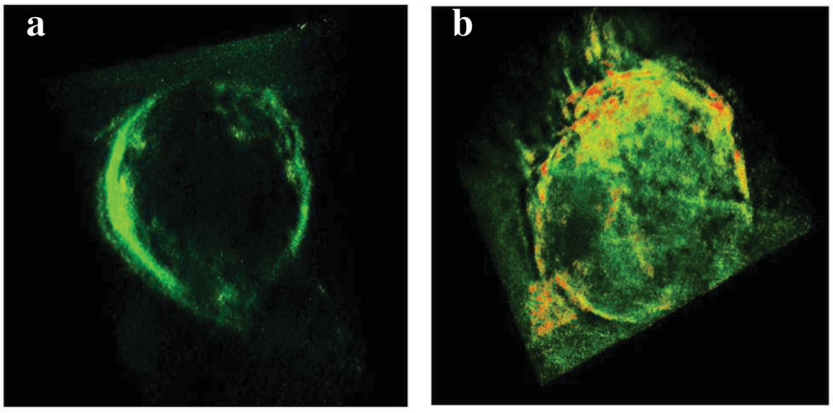

Figure 3. The 3D reconstructed tomography images of Hela cells. (a) control cells; (b) cells after incubation with TiO_2 NPs for 6 h (red color indicates the Ti signals).[40] Copyright 2013, Royal Society of Chemistry.

high-resolution transmission X-ray microscopy (TXM) to study the interaction between TiO_2 NPs with Hela cells. The 3D reconstructed tomography maps of cells treated by TiO_2 NPs showed intense Ti signals inside cells (**Figure 3**), further demonstrating the possibility of TiO_2 NPs as X-ray-sensitive probes for cell labeling.[40]

3.3. QDs

Fluorescent II-IV QDs are single crystals with several nanometers in diameter. With many attractive optical properties, such as high photoluminescence quantum yield, broad absorption coupled with narrow emission, and strong photostability, they have become an important fluorescent probes for cellular imaging.[37] Interestingly, since the high atomic number elements in QDs, e.g. cadmium(Cd), selenium(Se), tellurium(Te), are usually not present inside the cell, they provide a unique opportunity for developing X-ray sensitive nanoprobes. Corezzi et al. employed commercially available QD–secondary antibody conjugates to label two types of markers. One is β-tubulin, a protein associated with cytoskeleton microtubules and the other is cancer marker HER2 (human epidermal growth factor receptor 2) on the surface of SKOV3 human ovarian cancer cells. For intracellular labeling of β-tubulin, labeled cells were fixed with epoxy resin that was subjected to slicing. Slices of 60 nm in thickness were attached to the Si_3N_4 substrate for microXRF imaging. For surface labeling, cells were directly cultured on Si_3N_4. Pixel-by-pixel microXRF analysis of the Se element generates a topographical map of their intracellular distribution that reflects the locations of labeled proteins (**Figure 4**).[45]

3.4. Other Types of nanomaterials

Due to the excellent elemental specificity of X-ray spectroscopy for many elements, other materials containing various metal elements also have the potential to be explored as X-ray-sensitive nanoprobes. For example, Moronne developed lanthanide organo-polychelate conjugates as soft X-ray probes to target actin stress fibers and in vitro preparations of polymerized tubulin.[46] Yi et al. prepared Fe_3O_4@SiO_2 core-shell NPs to label human mesenchymal stem cells (hMSCs) for magnetic resonance imaging study. STXM images of intracellular Fe indicated even distribtuion of Fe_3O_4@SiO_2 NPs in hMSCs' cytoplasma, demonstrating the effectiveness of Fe_3O_4@SiO_2 NPs for stem cell tracking.[47] In addition, by using microXRF technique, subcellular localization and distribution of various kinds of metal elements were clearly observed.[48–51] For instance, Finney et al. found large-scale relocalization and extracellular translocation of cellular copper during angiogenesis.[48] Carmona et al. revealed the manganese accumulation in the Golgi apparatus of dopaminergic cells after exposure to manganese.[49] In the work of Aitken et al., cells treated with KP1019 [a kind of Ru(III) anticancer drugs] showed a high correlation between the Ru and P fluorescence signals, indicative of uptake of the drug into the nucleus.[50] Recently, Bresson et al. indicated that

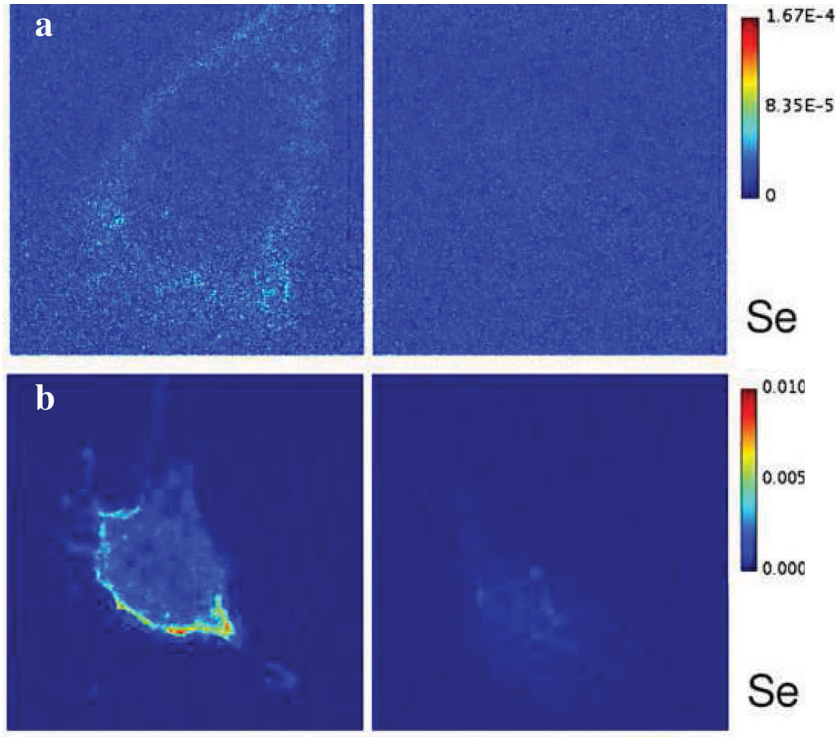

Figure 4. NanoXRF elemental maps of (a) thin (60-nm) sections of adherent human SKOV3 cells included in epoxy resin and mounted on Si_3N_4 grids and (b) human SKOV3 dehydrated cells. Left: β-tubulin (a) and HER2 (b) labeled by CdSe QDs. Right: control sample.[45] Copyright 2009, Elsevier.

upon exposure of BEAS-2B cells to cobalt, cobalt was homogeneously distributed in the nucleus and cytoplasm whereas zinc was more abundant in the nucleus, showing a modulation of intracellular zinc homeostasis.[51] The success in imaging these metal elements suggests that we could design nanomaterials containing these specific metal elements and use them as X-ray excitable probes in cellular labeling and imaging.

4. Summary

The rapid development in cell biology calls for highly demanding imaging technologies. While various microscopic techniques such as fluorescence, Raman and immunoelectron microscopy have been successfully employed in this regard to gain molecule-level understanding of cellular processes, it remains challenging to monitor multiple molecular targets with the nanometer-scale resolution. Recent advances in synchrotron-based X-ray imaging technologies have made X-ray microscopy a new tool for cellular imaging. They have shown unprecedented advantages including large penetration depths, high resolution and multiple imaging mechanisms. To realize biomolecule-specific labeling of cells, several types of X-ray sensitive functionalized nanoprobes have been successfully developed and show effective cellular labeling.

However, the development of X-ray excitable nanoprobes is still in its infancy. While X-ray microscopy potentially has the ability to discriminate multiple metal elements, there have been limited studies in this direction. Given the complexity of signal pathways in cells, which relies on the concerted action of ensembles of biomolecules and on networks of transiently interacting proteins, it is indeed important to simultaneously image the location of multiple proteins, and ideally, with the nanometer resolution. Since synchrotron-based X-ray microscopy has excellent elemental specificity, it is particularly suitable for simultaneous imaging of multiple cellular proteins by using a spectrum of nanoprobes with different metal elements. Nevertheless, it is technically demanding to synthesize nanomaterials with uniform sizes and high biocompatibility with different composition. The possibility of crosstalk of antibodies also poses a significant problem. All these studies require highly collaborative work that combines expertise in materials, chemistry, physics and biology. Despite these difficulties, the unique features and high potential of synchrotron-based X-ray microscopy offer great promise for localization of specific intracellular proteins and organelles. It is also envisaged that the development of more advanced synchrotron radiation facilities such as nanobeam projects and diffraction limited sources should provide unprecedented power for cellular imaging which, if properly implemented, should reveal new insights into the structure-function relationship.

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