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Imaging cells and sub-cellular structures with ultrahigh resolution full-field X-ray microscopy

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ABSTRACT

Our experimental results demonstrate that full-field hard-X-ray microscopy is finally able to investigate the internal structure of cells in tissues. This result was made possible by three main factors: the use of a coherent (synchrotron) source of X-rays, the exploitation of contrast mechanisms based on the real part of the refractive index and the magnification provided by high-resolution Fresnel zone-plate objectives. We specifically obtained high-quality microradiographs of human and mouse cells with 29 nm Rayleigh spatial resolution and verified that tomographic reconstruction could be implemented with a final resolution level suitable for subcellular features. We also demonstrated that a phase retrieval method based on a wave propagation algorithm could yield good subcellular images starting from a series of defocused microradiographs. The concluding discussion compares cellular and subcellular hard-X-ray microradiology with other techniques and evaluates its potential impact on biomedical research.

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1. Introduction

The development of new microscopy methods is a key element in the historical progress of biological and biomedical research. A landmark for each microscopy technique is the detection of individual cells and of their internal structure (Fischer et al., 2011; Marsh et al., 1971; Marton, 1941; Porter et al., 1945; Tanaka and Fukudome, 1991). For over one century, X-rays did play a fundamental role in biomedical research — but they were so far unable to image internal cell features in tissues. In a series of recent experiments, we achieved this important objective.

Our strategy was based on a combination of factors to improve the image contrast and spatial resolution. One of them was the use of highly bright and coherent X-rays emitted by synchrotron sources. The coherence specifically facilitated the task of focusing the radiation to improve the resolution. Furthermore, it enabled us to improve the image contrast by exploiting mechanisms (Cloetens et al., 2002; Hwu et al., 2002) based on the real part of the refractive index rather than on absorption (the imaginary part). These mechanisms – conventionally called "phase contrast radiology" – strongly enhance the contrast between soft tissues in biological specimens.

Our experiments used short-wavelength (~1 Å) X-rays capable to penetrate thick specimens in a natural state. On the contrary, previous microradiology tests were based on long-wavelength soft-X-rays in the "water window" (23–45 Å) that maximize the contrast between carbon-containing areas and water (Jacobsen et al., 2002; Kirz et al., 1994). With advanced soft-X-ray optics, subcellular structures could be observed in hydrated states (Larabell and Nugent, 2010; McDermott et al., 2009). However, soft-X-rays cannot penetrate specimens thicker than a few cells (≈10 μm) and are thus unsuitable for cell studies at the tissue level. This is a rather severe

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limitation (similar to visible light microscopy) that largely offsets the advantages of the high spatial resolution.

In a precursor experiment, we used hard-X-rays to image individual cells in a fresh and unstained state (Hwu et al., 2004) — the probed tissue thickness reaching several mm. Such tests included time-resolved experiments and tomographic reconstruction. However, they did not yet produce high-quality images with subcellular resolution.

We could finally reach this key objective by magnifying the X-ray images with Fresnel zone-plate objectives fabricated with state-of-the-art nanotechnology (Chu et al., 2008; Lo et al., 2007a, 2007b). These devices yielded a spatial (Rayleigh) resolution down to <20 nm (Chen et al., 2011a) and an image acquisition time below 50 ms (Chen et al., 2008a, 2008b).

We used staining to label and detect specific targets while locating proteins inside the cells and investigating sub-cellular organelles. This required a specific effort to develop suitable staining procedures since those used for other techniques, such as visible, confocal and fluorescence microscopies, do not fully match the needs of hard-X-ray microscopy. Specifically, staining methods for emission microscopy are not relevant to absorption or phase contrast X-ray images. Our staining/labeling method, based on immunocytochemistry, widely used for TEM (transmission electron microscopy), enabled us to investigate with nanometer resolution specific protein structures and distributions in two and three dimensions (3D). However, modifications of the staining materials and procedures were required because of the large difference in the specimen thickness for TEM and X-ray microscopy. Indeed, most of the heavy metal staining used for TEM is for thin (<1 μm) sections, and does not guarantee uniformity on our >100 μm specimens. We thus adopted and extended exposure to the staining solution.

Compared to previous soft-X-ray microscopy approaches, our method offers marked advantages. It penetrates much deeper into the specimen: intracellular details within >5 mm thick specimens could be clearly identified without critically decreasing the spatial resolution. As to hard-X-rays, previous tests (Hsieh et al., 2004; Jiang et al., 2010) could reach nanoscale resolution only by applying sophisticated phase retrieval methods to diffraction imaging. Recent developments in coherent diffraction imaging significantly simplified the image reconstruction; however, we reached subcellular resolution with no image processing. However, we did explore the use of a phase retrieval technique based on a wave propagation algorithm (Barty et al., 2000; Hsieh et al., 2004; Yin et al., 2007) to reduce or eliminate the need for staining. This approach yielded separate phase and amplitude images at the subcellular level.

As to possible future improvements, efforts are underway to achieve more versatile functional labeling and functional imaging on the nanometer scale. The current <30 nm lateral resolution of our zone plate objective is far from the theoretical diffraction limit and also from the estimated instrumentation limits. With even better zone-plate nanofabrication the lateral resolution – primarily determined by the smallest outer zone – can be further enhanced: outer zones ~20 nm were already achieved (Chen et al., 2008b, 2011b; Chu et al., 2008; Lo et al., 2007a, 2007b) yielding resolutions <20 nm. New high brightness X-ray sources will likely decrease the exposure time reducing for example the motion blur for live specimens. Furthermore, they would enable us to use higher-order diffraction modes (Yi et al., 2011; Yun et al., 1999) of the zone plates and further enhance the lateral resolution — although with a decreased signal and therefore a longer image acquisition time.

2. Materials and methods

2.1. Instrumentation

The experiments were performed on the beamlines 01A and 01B of the National Synchrotron Radiation Research Center (NSRRC) in Taiwan (Song et al., 2007), the 32-ID-D beamline of the Advanced Photon Source (APS, Argonne) (Shen et al., 2007) and the 7B2 beamline of the Pohang Light Source (PLS) (Baik et al., 2004). Specifically, tests with high spatial resolution were implemented on the NSRRC 01B and APS 32-ID-D beamline whereas the two other facilities were used for experiments with more limited resolution but faster image taking.

The high-resolution NSRRC and APS facilities are equipped with ultrahigh resolution transmission X-ray microscopes (TXM) including custom-designed microimaging systems with *ad hoc* fabricated Fresnel zone-plates (Chen et al., 2008b; Chu et al., 2008). The X-ray source is a 5-T superconducting wavelength shifter (SWLS) inserted along the 1.5 GeV NSRRC ring and an undulator at the 7 GeV APS ring.

The X-rays generated by these insertion devices are monochromatized by double crystal monochromators and then focused into the specimen by condenser lenses. The transmitted radiation is processed by the zone plate magnifying objective and projected into an array detector — consisting of a CCD camera with a 20× optical objective lens coupled to a scintillator. The total magnification of the zone-plate plus the visible light imaging system is 900–2400×.

The gold zone plates used for the present experiments were fabricated on thin (100 nm) SiN_x membranes and have ~1.5–3 cm focal length, a diameter of 85 μm, thickness up to >450 nm (corresponding to aspect ratios in excess of 15) and a 30 nm outermost zone width. To obtain phase contrast for low X-ray absorption materials, the microscopes are equipped with Zernike gold phase rings. The selected photon energy of 8 keV simultaneously optimizes the zone plate effectiveness and the contrast. The typical exposure time for a single image with 900× magnification is ~50 ms.

We used zone plates with 25 nm outermost zone width as the best compromise between efficiency and spatial resolution for this particular study. We measured the spatial resolution with the Rayleigh criterion (for the procedure, see Chu et al., 2008), using a standard test pattern whose smallest feature size was ~30 nm. Images such as those in Supporting Information Fig. SI1 confirmed that the smallest features are visible, corroborating the resolution measurements. The resolution measurements were performed both before and after the experiments.

Details about the two other experimental facilities, 01A at NSRRC and 7B2 at PLS, can be found in refs. Baik et al. (2004) and Song et al. (2007). The main difference with respect to the ultrahigh-resolution systems is that they use unmonochromatized X-rays, reaching more limited spatial resolution but gaining more than 3 orders of magnitude in time resolution compared to experiments with monochromatized X-rays. This is due to the fact that zone plates cannot be used with unmonochromatized X-rays: without zone plate magnification, the transmitted X-rays are directly detected by a cleaved $CdWO_4$ single-crystal scintillator and converted to visible images. These images are then magnified by an optical lens and captured and stored by a CCD camera. The resulting image taking is quite fast: for biological specimens with low X-ray absorption, a single image (typically 1600×1200 pixel, horizontal field of view (FOV) = 500 μm) can be obtained in 1 ms or less.

The specimens in both facilities are mounted on translation/rotation stages for precise positioning. The specimen can thus be moved along the X-ray beam direction, varying the specimen-detector distance from <1 mm to 1.2 m. This is specifically required for the phase retrieval method: a series of defocused images must indeed be taken at different specimen-detector distances. A similar method in the monochromatized facilities achieves phase retrieval in TXM.

2.2. Cell culture and staining

HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf fetal serum culture medium. EMT cells were cultured in Dulbecco's modified Eagle's medium and

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