Three-dimensional imaging of human stem cells using soft X-ray tomography

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Three-dimensional imaging of human stem cells using transmission soft X-ray tomography (SXT) is presented for the first time. Major organelle types—nuclei, nucleoli, mitochondria, lysosomes and vesicles—were discriminated at approximately 50 nm spatial resolution without the use of contrast agents, on the basis of measured linear X-ray absorption coefficients and comparison of the size and shape of structures to transmission electron microscopy (TEM) images. In addition, SXT was used to visualize the distribution of a cell surface protein using gold-labelled antibody staining. We present the strengths of SXT, which include excellent spatial resolution (intermediate between that of TEM and light microscopy), the lack of the requirement for fixative or contrast agent that might perturb cellular morphology or produce imaging artefacts, and the ability to produce three-dimensional images of cells without microtome sectioning. Possible applications to studying the differentiation of human stem cells are discussed.

1. Introduction

Pluripotent and multipotent human stem cells possess the extraordinary capacity to self-renew and generate specialized cells in response to appropriate environmental signals. As such, this class of cells is an invaluable research tool for regenerative medicine, which aims to replace or repair damaged tissue [1–3], as well as enabling disease modelling, whereby stem cells with specific genetic or functional defects provide an opportunity to interrogate human pathologies in vitro [4–7]. These fields have been bolstered with the identification of novel sources of pluripotent and multipotent cell populations from embryonic, adult and perinatal tissues, such as induced pluripotent stem cells [8] and human amnion epithelial cells (hAECs) obtained from term placentae [9,10]. Application of stem cells for regenerative medicine and disease modelling requires a robust understanding of the process of cellular differentiation. Knowledge regarding specific intracellular changes that occur during differentiation will assist in the development of desired stem cell progeny and progress research towards a better understanding of the nature of pluripotency. This knowledge would be greatly assisted by advances whereby cellular morphology could be imaged in three dimensions with minimal perturbation caused by sample preparation.

Traditionally, researchers have focused much of their attention on specific gene and protein markers to identify and characterize both mature cell populations and their immature progenitors. Expression of specific genes and proteins is used to predict cellular activity and function in mature cell types and to define mature cellular phenotypes. The differentiation of stem cells into their mature progeny is correlated with the suppression of genes and proteins related to self-renewal and pluripotency, and the increase in gene and protein expression specific for the mature cell phenotype. However, recently there has been a greater understanding that important and functional roles related to the differentiated state are...
reflected in other phenotypic characteristics such as cell size, cellular architecture and organelle number, size, shape and density. For example, it is well known that stem cell populations alter their shape, cytoskeleton and organelle composition during differentiation. For example, human mesenchymal stem cell commitment to adipocyte or osteoblast fate is influenced by both cell shape and cytoskeletal tension [11]. Similarly, cytoskeletal changes appear to be definitive for key stages in stem cell differentiation particularly in neural lineages [12]. Further, mitochondrial arrangement has also been shown to be a valid indicator of stem cell differentiation competence, possibly due to changes to metabolic activity required for lineage commitment [13]. Morphological changes that occur during stem cell differentiation have essential functions and can include the projection of cellular elements to form neurites that conduct electrical impulses between mature neurons, or cytoskeletal polarization during the formation of cuboidal lung epithelium. Therefore, in addition to gene and protein expression, there are myriad cellular changes that occur that affect cellular function that are currently difficult to quantify using current methodologies.

A greater understanding of the cytoskeletal and organelle composition and arrangement during stem cell differentiation would greatly assist efforts to develop lineage committed stem cell-derived populations for research, drug testing or cell therapy applications. A traditional method to visualize changes in cytoskeletal structure and organelle arrangement has been low spatial resolution analysis using standard confocal fluorescence light microscopy and confocal laser scanning microscopy, or high spatial resolution transmission electron microscopy (TEM), both of which require fixation and contrast agents that can alter morphology and introduce visual artefacts. While these methods have provided valuable information regarding cellular changes during differentiation, confocal fluorescence images have limited spatial resolution compared with TEM and require multiple antibody stains to provide an indirect overview of more than one aspect of cellular structure. On the other hand, TEM provides high-resolution two-dimensional information, but is limited by the harsh fixation and sectioning methods necessary and incompatibility with specific antibody staining. In addition, while it is possible to reconstruct three-dimensional tomographic images using two-dimensional electron tomography [14,15], this method is very time consuming and suffers as tissue is lost in the sectioning process and use of harsh fixatives and contrast agents [16].

Hard X-ray tomography is another technique that is used extensively to image biological samples. The most common applications of hard X-ray tomography are in the micrometre to millimetre resolution length scale (appropriate for Earth science, materials science and medical applications, for example). Although hard X-ray tomography instruments which achieve sub-100 nm resolution exist, biological specimens have very low absorption in the hard X-ray region, imparting challenges in using this technique for their analysis. One approach to bypass this limitation involves 200–500 nm ultramicrotome sectioning for the visualization of intracellular components [17]; however, this laborious process has prevented widespread application of the technique. A number of researchers are pursuing phase imaging to overcome this limitation [18–20] and it will be interesting to track the development of the hard X-ray tomography technique and see if it can successfully be applied for cellular imaging at resolutions relevant to resolve organelles.

Transmission soft X-ray tomography (SXT) is a new technology that may overcome the spatial resolution and image contrast limitations of the other microscopies for studying stem cells providing high spatial resolution imaging, in all three dimensions, of fine-scale structural components in thick samples (up to approx. 12 μm, as described below).

SXT represents a much higher resolution version of the familiar computed tomographic clinical imaging methodology, providing spatial resolution on the order of 50 nm, a pertinent spatial observation window previously inaccessible to researchers observing intact cells, between confocal fluorescence and TEM. The soft X-ray wavelengths employed in SXT are approximately 2 nm, and do not limit the imaging resolution, which is instead limited by the nanofabricated X-ray optics used in the instrument. These optics have been demonstrated with capabilities down to 10 nm and better [21,22]. In addition to resolution, an important capability of SXT is its ability to distinguish subtle differences in X-ray linear absorption coefficients (LACs). Different materials, and thus different biological intracellular components, have characteristic LAC values, so the ability to quantitatively image the three-dimensional distribution of LAC values can be used to discriminate between biological intracellular components. The chemical compositions of organelles vary widely depending on their class, with vesicles, for example, a combination of water and lipid molecules, while mitochondria consist of dense granules and a proteinaceous matrix. These variations translate to clearly distinguishable differences in X-ray absorption, measured as an X-ray LAC [23,24]. Relying on LAC imaging in this manner renders unnecessary the harsh fixation methods and contrast agents of traditional techniques such as TEM, which often dehydrate, cross-link or otherwise modify intracellular structures; for SXT, samples are simply flash frozen prior to imaging. Equally, fluorescent antibody imaging agents used in confocal microscopy are not essential as the cell and intracellular structures are directly imaged, providing arguably a more accurate representation of intracellular morphology. While SXT presents intense sources of radiation to specimens, the appearance of damage to biological materials in the images at the approximately 50 nm resolution presented here is avoided by maintaining the samples at cryogenic temperatures during imaging [25,26].

SXT has hitherto been applied mainly to inanimate materials [27], viruses [28] and simple organisms such as protozoa, unicellular eukaryotes and fungi [24,29–33]. Recently, a few studies have captured tomographic images of mammalian cells, typically small erythrocytes or leucocytes [34–37], including human cells [38,39] but to our knowledge there has been no literature reporting SXT of more complex human cells, such as stem cells of any kind.

Here, we report the first direct imaging and contemporaneous examination of cytoskeletal and organelle morphology in human stem cells and their differentiated progeny with SXT. Additionally, we show that traditional antibody staining for specific proteins can be combined in stem cells with SXT technology to visualize the cellular localization of proteins concurrently with three-dimensional direct imaging of intracellular components. While antibody labelling for SXT has been demonstrated [38,40], this is the first demonstration in human stem cells. Furthermore, we demonstrate the successful application of SXT technology in two stem cell types and report on the advantages of this new imaging modality to the field of stem cell biology. We argue that the ability to image
intracellular components of human cells with this new approach will have a major impact on a plethora of research questions in the field of cell biology, including the study of fertilization, cell apoptosis, mechanisms of viral infections, liposomal and protein diseases.

2. Material and methods

2.1. Culture of human amnion epithelial and human embryonic stem cells

All experiments were performed under guidelines set by the Monash Human Ethics Committee, Monash University. Experiments were approved by the Monash Human Research Ethics Committee as part of the research project application ‘Establishment of a tissue bank and associated database for use by the Centre for Women’s Health Research.’ No. 01067B. hAECS were isolated as previously described [9]. Briefly, placenta were obtained from women with uncomplicated pregnancies undergoing elective caesarean section at term. The amnion membrane was manually stripped from the chorion membrane and hAECS enzymatically removed from the amnion by two, 1 h digestions in Trypzean (Sigma-Aldrich). Trypzean was inactivated by soybean trypsin inhibitor (Sigma-Aldrich) and hAECS collected by centrifugation. hAECS were cultured in either Small Airway Epithelial Growth Medium (Lonza) to induce differentiation into the lung epithelial lineage or DMEM/F12 10% FBS to maintain hAECS in an undifferentiated state for up to 28 days without passage.

Human ESCs (H9 line, WiCell) were cultured as previously described [41]. Briefly, hESCs were grown in co-culture with 2 × 10^5 mouse embryonic fibroblasts per cm^2 in DMEM/F12 media supplemented with 20% Knockout Serum Replacement, 1% Glutamax, 1% Insulin-Transferrin-Selenium-X, 1% non-essential amino acids, 0.5% penicillin/streptomycin and 0.2% β-mercaptoethanol and 10 ng ml⁻¹ bFGF (Invitrogen). Human ESCs were passaged enzymatically every 4–5 days upon reaching confluence at a ratio of 1:5 using Trypzean Select (Invitrogen).

2.2. Sample isolation and preparation

All cells were enzymatically disassociated to form a single-cell suspension from culture using Trypsin 0.05% for 1–5 min at 37°C and were then washed in PBS before fixation in glutaraldehyde for 5–10 min. As discussed earlier, SXT produces images from the contrast of X-ray absorption and fixation is not required. For this study, however, as cell samples were generated in Melbourne, Australia, both quarantine regulations and transportation times to the synchrotron location in Berkeley, CA, mandated treatment with fixatives to preserve cyto-architecture.

Upon arrival at the Lawrence Berkeley National Laboratory, cell suspensions were passed through 40 μm filters to exclude aggregates of multiple cells that would otherwise cause blockages and prevent cells advancing to the narrow end of glass capillaries. This process also removed a limited number of large irradiated fibroblasts present within hESC preparations. Samples were diluted to a low concentration (0.5–10^5 cells ml⁻¹). Cells were pipetted into thin-walled (200 mm), pulled glass capillary tubes and advanced to the narrow end (approx. 15 μm internal diameter) by gentle centrifugation (400–600 g) for 1–5 min. Glass capillaries containing specimens were then mounted immediately within the rotation-capable cryogenic stage of the XM-2 soft X-ray microscope on beamline 2.1 at the National Centre for X-ray Tomography at the Advanced Light Source (ALS).

2.3. Gold nanoparticle immunocytochemistry

To determine the distribution of specific proteins within individual, differentiated cells, single cells were harvested from confluent hAECS cultures maintained in SAGM for 28 days and labelled with an antibody specific for the Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein. Briefly, cells were fixed in 4% paraformaldehyde in PBS for 15 min at 4°C and permeabilized with 0.1% Triton-X-100 in PBS for 5 min at room temperature. Cells were incubated with rabbit anti-CFTR primary antibody at 1:1000 (Cell Signaling no. 2269, Invitrogen) and then with 1:1000 Alexa Fluor 488 FluoroNanogold-conjugated secondary antibody (Molecular Probes no. A-24922, Invitrogen), each for 1 h at room temperature. Visualization of CFTR-conjugated gold nanoparticles was enhanced using a LI Silver enhancement kit (Invitrogen).

2.4. Transmission soft X-ray microscopy

Samples were flash frozen by a stream of liquid nitrogen-cooled helium and maintained at cryogenic temperatures throughout the imaging. All imaging was done at the ‘XM-2’ instrument of the National Center for X-ray Tomography, at Beamline 2.1 at the ALS in Berkeley, CA. In that instrument, a synchrotron bend magnet produces X-rays which are directed by a mirror to a Fresnel zone plate. The x-ray optic was placed on the outside of the capillary tube as fiducial markers. The sample out of the field of view) and IMOD 4.1 software was used then imaged onto a back-thinned CCD detector using a Fresnel zone plate objective optic with outer zone width of approximately 50 nm (figure 1). The two-dimensional resolution of zone-plate-based X-ray microscopes is well known to be approximately equal to the outermost zone width [42]. The exact three-dimensional resolution was dependent on additional factors, including sample contrast, number and distribution of angles collected, and sample alignment. Using test samples and the noise-compensated leave-one-out method [43], the three-dimensional resolution of the instrument was found to be close to the two-dimensional resolution of 50 nm determined by the zone plate used (DP 2010, personal communication).

X-ray image datasets comprised a series of 90 projection images, sequentially acquired every 2° as the sample was rotated through a total of 180°. For a given zone plate resolution, the field of view is determined by the number of pixels. The camera used had 512 × 512 pixels, which were binned by 4 in each dimension, with each pixel having a dimension of 32 nm on a side, giving a field of view of approximately 16 μm. Exposure times between 150 and 300 ms were used. Images were normalized by bright fields (collected before and after the projection series by moving the sample out of the field of view) and IMOD 4.1 software was used for manual alignment of the projection images to a common frame of reference [44] using gold nanoparticles which had been placed on the outside of the capillary tube as fiducial markers. Tomographic reconstructions were performed using iterative reconstruction methods [45,46] with segmentation, surface visualization and volume rendering done using AMIRA software (FEI Visualisation Sciences Group, Burlington, MA, USA). The colour and transparency of the colourmap for the rendered volume was adjusted to make intracellular structures visible.

Determining the boundaries of various intracellular components (such as organelles) is a process known as ‘segmentation’ (or digital labelling) and can be done semi-automatically using tools in the segmentation editor interface of Avizo. In the case of a number of the intracellular components, the three-dimensional equivalent of a ‘magic wand’ in the Photoshop software was used, whereby one voxel (three-dimensional pixel) was selected, along with a positive and negative greyscale threshold. The boundary of the object expands from the starting voxel to encompass all adjacent voxels that fell within the selected greyscale threshold. By choosing thresholds appropriately for different classes of intracellular components, groupings of
intracellular objects with the same voxel thresholds could be segmented (digitally labelled) serially using a single click within the segmentation editor interface.

Additional background information on both the experiment and image processing for SXT has recently been published [47]. We note here that in general, biological samples must be less than 12 μm in thickness to allow sufficient penetration by soft X-rays to achieve good images—for thicker samples, the overall %Transmission of soft X-rays in the water window energy range (280–530 eV) through the sample drops below 10% such that the count level on the detector is very low. This in turn leads to noisy and artefact-corrupted reconstructed three-dimensional images. There are a number of studies reported where hard X-rays are used for tomographic image reconstruction that penetrate tens of micrometres into tissues and cell clusters, but these methods achieve much lower spatial resolution (micrometre range) than using the SXT method described here [48–50].

The diameter of higher order eukaryotic cells, such as mammalian cells varies considerably depending on cell type and culture conditions, and remains a limiting factor for analysis by soft X-ray microscopy because of the upper limit for cell thickness for SXT of approximately 12 μm. SXT has been performed on adherent cells grown on TEM grids [51,52], which allows for cells that are larger than 12 in two of the dimensions, but not in thickness. While this allows larger samples to be imaged, it does limit the angular range over which projection images can be collected, which can reduce the quality of the final three-dimensional reconstruction.

At XM-2, SXT microscopy is performed on samples loaded within glass capillaries, so adherent cultures must be first disassociated and placed in suspension. The stem cells used in this study were grown in adherent conditions on extracellular matrix substrates, and enzymatic disassociation with trypsin was used to generate suspension cultures. While this has the disadvantage of disrupting certain morphologies of monolayer cultures systems, it has the benefit of reducing cellular dimensions to facilitate loading into the glass capillaries. Human stem cells are typically small and have high nuclear to cytoplasmic ratios [53]; however, this processing was crucial for enabling these cells to achieve dimensions that approached and fell below 12 μm in diameter.

The absence sample damage at the photon energies was used and was confirmed by comparing images at 0° and 180° and horizontally flipping one of the images. Direct comparisons could then be made and for all samples no changes were observed, indicating no sample damage at the resolution used (data not shown).

2.5. Transmission electron microscopy and area measurements

Cells were fixed by adding 0.2 ml of fixative (6% paraformaldehyde and 5% glutaraldehyde in 0.2 M cacodylate buffer) at room temperature for 1 h. After fixation, cells were centrifuged at 3200 r.p.m. for 10 min at 30°C, and washed with 0.1 M cacodylate buffer. After fixation the samples were postfixed with 1% osmium tetroxide, dehydrated in a graded series of ethanol and embedded in Epon. A total of 50–70 nm thick sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM 109 transmission electron microscope.

The ‘magic wand’ selection tool in Adobe Photoshop CS5 was used to encircle each organelle within digital files of both TEM
images and cross-sectional area dimensions were then recorded. Area values were converted from a pixel to a real value based upon scale bar measurements. To compare these measurements with those from SXT images, a single digital slice can be extracted from the reconstructed three-dimensional volume as a two-dimensional image, and then areas can be calculated based on this image as described above for TEM. All organelles within both TEM images were measured and collated into organelle categories by visual examination. SXT intracellular objects were collated using these methods, but with additional reliance on the measured LAC values within each organelle, as previous publications have shown that LAC values can be used to separate distinct organelles [22]. All graphical representations were generated using the PRISM program (GraphPad Software, Inc, CA, USA; figures 3 and 4), with data displayed as mean ± s.e.m., *p < 0.05, **p < 0.01, ***p < 0.001, using one-way ANOVAs and the Kruskal–Wallis non-parametric test with Dunny post-test. Raw data was found in electronic supplementary material, S5.

3. Results

3.1. Cell preparation

As described in the Material and methods section, specimens imaged by the XM-2 soft X-ray microscope were contained within fine-walled pulled glass capillaries (figure 1a,b), which were mounted before the detector between the microscope condenser and objective (figure 1c,d). Once cells were delivered to the appropriate position, sample loaded capillaries were then mounted for imaging by the XM-2 (figure 1d).

3.2. Soft X-ray imaging and three-dimensional reconstruction

Representative single two-dimensional images of three of the 12 total hESC (figure 2a–c) and 12 total hAEC-derivatives after X-ray imaging are shown (figure 2d–f). For each cell, this kind of two-dimensional image was repeatedly collected 90 times as mounted capillaries were rotated in 2° steps. Acquired two-dimensional datasets for each cell were tomographically reconstructed to generate a single three-dimensional volume, consisting of the measured LAC value for each point in three-dimensional space. This three-dimensional volume can be visualized in a number of ways. One of these ways is by taking ‘digital sections’ through the volume. Several of these one-pixel-thick slices for an individual hESC (bi–v) and hAEC (vi–v) are shown, with an example tracing (segmentation) of organelles, outlined in yellow and indicated by arrows.

Figure 2. Three-dimensional reconstruction methodology. (a–c) Representative two-dimensional digital sections from three hESCs and (d–f) three hAECs shown in greyscale. After tomographic reconstruction, digital sections of the three-dimensional volume can be visualized. A series of sections for an individual hESC (bi–v) and hAEC (vi–v) are shown, with an example tracing (segmentation) of organelles, outlined in yellow and indicated by arrows. Full three-dimensional volume visualization of a hESC (g) and hAEC (h) are shown with false-coloured organelles. (Online version in colour.)
referred to as a lookup table) is used to map LAC values to the colours or grey values that are seen on the screen to aid in the process of segmentation. Low LAC values correspond to darker grey colours (black being the lowest), whereas higher LAC values correspond to lighter grey colours (white being the highest).

Another way to visualize the volume is to show the boundaries of relevant structures within the sample, and then display a three-dimensional rendering of the full volume. This was done for figure 2g–h and three-dimensional files found in electronic supplementary material, figures S1 and S2, after image segmentation was completed as described in the Material and methods section. After segmentation, each grouping of intracellular objects can be volume rendered or surface rendered using a different colour. If volume rendering is used, a colourmap that includes variations in colour and transparency can be used; generally, higher LAC values are shown as more opaque. Videos showing a series of slices through the reconstructed volumes of all 12 hESCs and hAECs imaged are shown in electronic supplementary material, figures 3 and 4, respectively.

3.3. Volume and density evaluation of human stem cell samples

Volume and density evaluations were performed for the hESC images acquired as opposed to hAEC-derivative images, due
to the considerably greater quantity of literature available from alternative imaging methodologies of hESCs for comparative purposes. Firstly, several intracellular objects visualized by SXT may be assigned *a priori*, due to their well-characterized location and morphology. This was used for identifying the nucleus as it was the largest intracellular structure (figure 3a, blue arrowhead), and the organelles within it, that were obviously nucleoli (figure 3a, blue arrow). Both of these organelles were assigned false colour (blue), with colour density directly proportional to organelle density (figure 3f). Panels (a–d) show visualizations of digital slices through the volumes, with brighter colours indicating higher LAC values. Figure 3f shows the volume rendering of the cell after segmentation of the intracellular objects of interest.

Many intracellular objects were not classifiable in this manner, as their size and location were indistinct. To assist with object assignment, we performed accurate measurements of each object’s volume and average LAC values from the segmented SXT images. In cases of objects that were especially complex, or in cases where the difference in LAC between adjacent structures was very small, it was necessary to manually trace the outlines of individual objects in each two-dimensional digital slice that constituted the three-dimensional image. This was done based on visual inspection of the reconstructed images. As mentioned earlier, the apparent contrast in slices through the reconstructed images (figure 3a–d) was a direct representation of LAC values. To confirm that the location of the boundaries selected by eye were optimal, these boundaries were overlaid on images representing the gradient magnitude of the three-dimensional images. Gradient magnitude images have their highest values at locations of the fastest change in LAC value in the image, which corresponds to the boundaries between objects. The manually traced boundaries were fine-tuned to be in line with the boundaries visible in these gradient magnitude images (figure 3e).

Once segmentation of all intracellular objects within the reconstructed three-dimensional SXT cell image was completed, precise LAC, volume and surface area values could be calculated for each individual object, and clusters of objects could be determined (figure 3g,h). The localization of the nucleoli within the nucleus enabled their identification to be made with confidence. Remaining intracellular objects were distributed across a spectrum of LAC values, with low LAC represented by dark contrast areas (figure 3b, red arrow), and high LAC as intensely white (figure 3c,d, black and green arrows).

LAC and volume measurements were then used to classify these remaining objects, using a combinatorial approach.

**Figure 4.** Organelle classification. (a,b) TEM images of hESCs were captured and used as references for comparisons to SXT images to assist with organelle classification. TEM images clearly show the presence and characteristics of potential organelle groups such as mitochondria (black arrows), lysosomes (red arrows) and unclassified objects, possibly small secretory vesicles or ribosomal clusters (green arrows). (c) Matching colour arrows showing examples of similar objects in three-dimensional rendered SXT hESC image. (d) Area values were calculated from SXT (open bars) and TEM (striped bars) images and compared; organelles which remained unknown were classified based on the closest match to TEM references and original colouring indicated by X-axis text highlighting. *p < 0.05, **p < 0.01, ***p < 0.001. (Online version in colour.)
The largest unclassified objects were false-coloured red (figure 3f, red arrows) and were seen to exhibit a tight distribution of low LAC values compared with other objects (0.008 ± 0.0002 μm\(^{-3}\), figure 3g). The remaining objects (with higher LAC values) were separated into two groups based on volume as two distinct groupings were evident, with the larger 0.288 ± 0.008 μm\(^{-3}\) false-coloured black and smaller 0.043 ± 0.007 μm\(^{-3}\) false-coloured green (figure 3f). Statistical separation based on volume measurements was observed between these black and green objects (figure 3h) and additionally LAC values demonstrated significance between most object groups (figure 3g). Despite confident separation of SXT identified intracellular structures into distinct categories, further assessment was required to provide confidence in an accurate classification of each object category into various organelle types.

It is important to note that, in this study, cell samples were lightly fixed with glutaraldehyde to acquiesce with US customs importation requirements. Aldehydes and other fixatives contain carbon molecules that bind to amine groups, cause the aggregation of molecules within cells and reduce lipid content. Although fixatives are reversible upon washing in buffers and aggregation of molecules within cells and reduce lipid content. Although fixatives are reversible upon washing in buffers and

### 3.4. Organelle characterization and comparison to transmission electron microscopy

As this report is the first to image human stem cells with SXT, and few reference data exist to assist with the assignment of intracellular structures to various organelle categories from detailed volume and LAC values. Accordingly, TEM images of undifferentiated hESCs were obtained and used as objective reference material for comparison with the SXT data (figure 4a,b). Morphological characteristics and a wealth of reference literature [54–57] assisted with the classification of organelles within TEM images, with mitochondria (black arrows), ribosomes (green arrows) and vacuoles (red arrows) appearing distinct and clearly identifiable (figure 4a,b). TEM images, while only two-dimensional, allowed for generation of approximate cross-sectional area values for each organelle at the representative serial section that was acquired. This was done similarly for digital slices through the SXT three-dimensional reconstructed hESC images, to provide a cross-sectional area measurement for each organelle and facilitate SXT and TEM comparisons.

Surface area dimensions of red objects acquired by SXT (1.00 ± 0.2389 μm\(^2\)) correlated closely with those of lysosomes seen in TEM (0.870 ± 0.2644 μm\(^2\)) images. While these measurements were not significantly different (p > 0.05), they were significantly different in size to all other potential organelle types that were evaluated (figure 4d). As such, the red objects identified by SXT imaging are likely to be lysosomes, or a similar organelle type. The most numerous SXT intracellular objects, coloured black, were found to be similar in size, distribution and surface area to mitochondria; 0.252 ± 0.017 μm\(^2\) (SXT) and 0.107 ± 0.010 μm\(^2\) (TEM). SXT objects coded green were tentatively assigned as secretory vesicles as they were equivalent in size (0.050 ± 0.006 μm\(^3\)) to observed secretory vesicles in TEM images (0.024 ± 0.001 μm\(^3\)) and were not statistically separable (figure 4d).

Overall, intracellular components could be separated into distanced groupings based on LAC and volume measurements from SXT datasets. These object groupings demonstrated significant differences to each other in regards to cross-sectional area, number and intracellular distribution (figure 4d). Groupings of intracellular objects identified by SXT correlated robustly with various organelle classes identified by TEM and were thus assigned with confidence, such as the nucleus, nucleolus and mitochondria. For other groupings, such as red objects, their classification as lysosomes or another similar organelle type (i.e. large vacuoles) is likely, but requires additional studies to confirm. One grouping of intracellular objects that were not confidently assigned to an organelle type, may possibly represent small secretory vesicles or ribosomal clusters, and differences in cross-sectional area between the SXT and TEM preclude definitive classification at present.

### 3.5. Gold conjugated secondary antibody labelling of cellular structures

One major advantage of SXT imaging is that, while three-dimensional imaging and identification and characterization of specific intracellular components can be performed without stains or labels, traditional antibody staining can be used for visualization of the three-dimensional distribution of specific proteins, without sacrificing the other information. Immunostaining can complement SXT to remarkably increase the information gathered with this technology. As proof of concept, we targeted the CFTR protein, which is associated with genetic lung disease. CFTR protein was labelled with a gold nanoparticle-conjugated monoclonal antibody, and the density signal was enhanced using LI silver. The gold/silver nanoparticles are significantly denser than the surrounding cellular components, providing a marked differentiation between the nanoparticles and biological densities, making them easy to segment and visualize. The advantage of this system is that specific localizations of proteins of interest can be identified and related to the location of cellular components such as the nucleus, mitochondria, lysosomes and cell membrane. In this study, we found that differentiated hAEC cells synthesized CFTR protein that was localized primarily on the cell membrane, expressed in a polarized manner. However, in undifferentiated hAECs, CFTR protein was not observed. These data suggest that following differentiation of hAECs from an immature stem-like phenotype to a mature lung epithelial cell phenotype, cells produce the functional CFTR protein, which is actively transported to the cell membrane, and that the cells undergo polarization similar to what is observed in cuboidal lung epithelium, confirming previous studies showing increased CFTR gene and protein expression [58] associated with this phenotype. Soft X-ray imaging and three-dimensional reconstruction facilitated the observation of protein polarization in our differentiated cells, whereas other two-dimensional imaging methodologies would not have been suitable for this analysis.

Figure 5 shows the results of this work. Figure 5a shows a digital slice through the reconstructed volume. Figure 5b,c shows three-dimensional volume renderings of the cellular and organelle structures, and the gold nanoparticle-conjugated
antibodies, respectively. Figure 5 shows an overlay of the gold nanoparticle-conjugated antibodies and the cellular structures, which provide information on intracellular organelle abundance, size, shape and localization, as well as information on the localization and abundance of specific proteins.

4. Discussion

This study is the first to show that soft X-ray tomographic imaging can be performed on human stem cells. We provide proof of concept for the use of this novel technique to provide very rapidly high-resolution three-dimensional images and quantitative data via direct analysis of intracellular organelles and other components. The limitations of the technique was that sample penetration is limited to approximately 12 μm, so only single cells could be interrogated and these needed to be cryogenically frozen to prevent photo-oxidative damage by the soft X-ray beam. Nevertheless, the imaging can be achieved using unfixed cells without the use of contrast agents.

We have demonstrated SXT provides a combination of quantitatively measured LAC and volume values for organelles within cells, along with their number, shapes, internal structures and distribution. Further, by using this objective data, we have shown that the identities of several organelle types are distinguishable. Overall, for this study, digital two-dimensional slices from the three-dimensional SXT dataset were evaluated in order to enable the direct comparison of organelle sizes in the two-dimensional TEM slices. While this methodology is not ideal, it did confirm that SXT can be used to image and identify intracellular organelle types based upon LAC and volume measurements. Further studies need to be performed to confirm these results, and to provide additional confidence for categorization of object groupings.

SXT data can provide valuable information regarding intracellular changes that occur during lineage commitment and provide a novel direct imaging approach to assess cellular functions. For example, mitochondria number, size and distribution are known to fluctuate in response to cell proliferation activity, while lysosome changes can be used to distinguish stem cells from lineage committed cell types. While traditional methodologies have provided us with a basic understanding of these differences, SXT provides a much more detailed understanding, facilitating the development of much more accurate assessments and visualization of cell differentiation processes.

We have also shown in a proof of principle study that the direct imaging of SXT can be combined with indirect traditional methods of immunostaining, to provide a powerful tool that can identify proteins of interest within the three-dimensional intracellular environment. Relevant to the example presented here, this technique may be used to measure the effect of cystic fibrosis drugs on the misfolding and Golgi degradation of CFTR protein. Indeed, the combination of SXT with immunohistochemistry has a remarkable breadth of applications and advantages, enabling, for example, specific proteins associated with intracellular vesicle trafficking, mitochondrial biogenesis or the proteasomal degradation system to be studied concomitantly with organelle size, position and physical composition. Assessment of these processes in perturbed scenarios (i.e. genetic defects or infection) could provide a clearer understanding of underlying mechanisms at a cellular level, as SXT alone has demonstrated recently [35].

We chose to analyse human stem cell populations due to their potential clinical applications for regenerative medicine. An understanding of the fine-scale structural physiology of stem cells is important, as the structures, numbers and ratios of organelles are known to change during differentiation to...
terminal identities. This study demonstrated that such aspects can be studied in exquisite detail using SXT. In addition, naïve stem cells and their differentiated progeny could be compared with pathological counterparts to assess key disease processes at the cellular level. For example, Huntington’s disease involves disruption to rates of mitochondrial fusion and fission [59] and the shape and quantities of mitochondria are quantitatively rendered using SXT. Additionally, Huntington’s, Alzheimer’s and Amyotrophic lateral sclerosis are some of the many human disorders involving protein misfolding and formation of intracellular aggregates, within the resolution range of SXT. The formation and distribution of these aggregates could be mapped over time to assist with understanding the aetiology of these pathological cellular phenotypes.

Future studies would greatly advance the utility of this immensely powerful technology by evaluating the breadth of interspecies organelle LAC and volume variation across commonly studied eukaryotic and prokaryotic species. Indeed, work towards this objective has been initiated recently [51]. With the collection and analysis of a larger body of LAC and volume datasets for a range of cell types, one can envisage future studies, whereby automated classification and image reconstruction allowing identification of organelles in an unambiguous manner, abrogating the present need for comparative studies with TEM images or a priori grounded decisions based upon organelle size, shape and location.

While this study comprised a limited number and type of cells analysed due to beamtime availability, this study has established a base protocol for SXT imaging analysis of human stem cells that will facilitate research of basic cell biology and the changes in cellular architecture throughout stem cell differentiation. These investigations on human stem cells shed new light on a multitude of intracellular structures contemporaneously, through the generation of highly informative images of cellular architecture hitherto unattainable by conventional imaging techniques.

**Competing interests.** We declare we have no competing interests.

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**References**


