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PERSPECTIVE

Compact Cell Imaging Device (CoCID) provides insights into the cellular origins of viral infections

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1. Overview

Viral diseases are not only an important cause of mortality, they also carry a significant social and economic cost to humanity. For example, the cost of influenza virus care in 2018 was estimated to be about €29 billion [1] or 2% of the healthcare costs of the EU. Other viruses, such as the hepatitis C virus (HCV), which has an incidence of 8.7 per 100 000 people in the EU [2], can cause a lifelong infection and is a major cause of liver cancer. Another hepatitis virus, the hepatitis E virus (HEV) causes acute viral infection worldwide, with an increasing incidence in Europe since 2010 [3]. The current severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) global pandemic, which has caused a high number of deaths and infections, will probably lead to a fifty-fold or more increase in flu healthcare costs in Europe [4]. While viruses are mostly known for their ability to cause disease, some viruses, such as the herpes simplex virus 1 (HSV-1), can be used to kill cancer cells [5, 6]. Altogether, viruses have an important impact on human health, and the costs of developing antiviral therapies and virus-mediated cancer therapy are considerable, in terms of economic, physical, social, and mental well-being.

An analysis of three-dimensional biological cell samples is critical to understand the mechanisms of viral disease and for the development of novel therapeutics. Soft x-ray microscopy (SXM) is a unique technology that can image whole intact cells in 3D under normal and pathological conditions without labelling or fixation, with high throughput and spatial resolution [7–9]. The main challenge of SXM is that the photonic illumination required for imaging is currently only available at five football-stadium sized facilities, called synchrotrons [10], and only a tiny fraction of the infectious disease research community has been able to access this imaging modality.

The new European Union Horizon 2020 project called CoCID, the 'Compact Cell Imaging Device,' which started in January 2021, proposes to address this challenge by the development of lab-scale SXM for fast and inexpensive three-dimensional imaging of whole cells that can readily be performed in a laboratory. The capabilities of this compact imaging device, combined with complementary light and electron microscopy approaches, will be demonstrated through a series of virology use cases to generate new scientific knowledge about the viral life cycle and host cell response to viral infection. Use cases are chosen to cover viruses utilizing different genetic materials, such as RNA for SARS-CoV-2 and DNA for herpes viruses, the transmission of viruses (hepatitis E) between species, and the development of antiviral drugs (for hepatitis C). The project specifically focuses on the mechanisms of virus–cell interactions that can be used in the development of antivirals and oncolytic viral therapies.

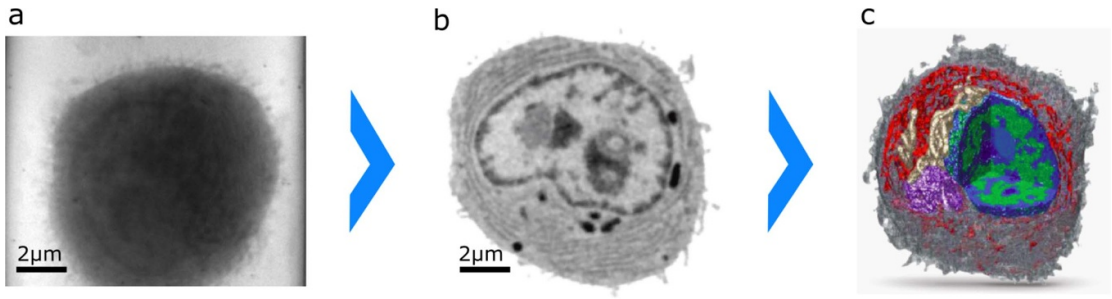

Figure 1. 3D tomography of a whole lymphocyte cell using SXM acquired at Advanced Light Source National Centre for X-ray Tomography. The sample is rotated in front of the soft x-ray beam. 2D projection images (a) are obtained at each rotation angle. These projections are used to reconstruct volume of a cell. (b) A virtual slice through a reconstructed volume. Based on the x-ray absorption values, segmented organelles are rendered in 3D (c). The cellular ultrastructure is clearly visible, illustrating the high natural contrast attainable from SXM.

2. CoCID concept and approach

The overall CoCID concept is centred on providing virologists with a next-generation imaging device, which, through increased penetration and depth of focus, as well as through high natural contrast and sensitivity to organelle density (including virus-related organelles), will produce higher-fidelity ultrastructural images of whole intact cells. These insights will, in turn, help increase our understanding of the links between the structural reorganisation of cells and the mechanisms of viral entry, replication, assembly, and egress in cells. CoCID will provide this valuable imaging capability in the form of a compact lab-scale device that will greatly improve the accessibility of SXM for the virologists' consortium within the CoCID project as well as the broader disease research community worldwide.

At synchrotron-hosted microscopes, the capabilities of SXM have already been proven to great effect in virology for the imaging of vaccinia [11, 12], HSV-1 [13–15], hepatitis C [16], reovirus [17] and SARS-CoV-2, in combination with cryo focused ion beam scanning electron microscopy (cryoFIB/SEM) [18]. In CoCID, the breadth and depth of specific virology studies that have already utilised synchrotron SXMs will be expanded, while at the same time, validating and revolutionising the utility of the lab-based SXM prototype.

2.1. SXM

SXM uses x-rays in the 'water window' that extends from the K-absorption edge of carbon to the K-edge of oxygen, that is from about 282 eV ($\lambda = 4.4$ nm) to 533 eV ($\lambda = 2.3$ nm). Water is transparent to these x-rays, but organic molecules are absorbent. Therefore, these x-rays can be used as the basis for microscopy of whole cells in their near-native (frozen) state, without the need for any contrast-enhancing agents. A 3D tomogram with a resolution between 25 nm and 60 nm (full pitch) is produced by rotating the cell over a range of angles, and acquiring an image at each tilt angle [9, 19, 20]. The concept is equivalent to a medical CT scan applied at the nanoscale, as shown in figure 1 (adapted from [21]). Similarly to Hounsfield units in medical CT, cellular organelles within the cell can be distinguished from each other by their respective x-ray linear absorption coefficient values. While great progress has been made over the last two decades in developing SXM as an imaging technique using synchrotron-hosted microscopes [9, 12, 22, 23], only two laboratory x-ray microscopy systems have been reported so far [24–26]. The CoCID concept is novel, insofar as, until now, no commercial lab-scale SXM has been available that is capable of delivering the necessary image quality and throughput required by the biomedical community.

2.2. Lab-scale SXM

The biggest challenge in developing a compact SXM is the production of a stable illumination source of soft x-rays with sufficient flux to image cells within a reasonable timescale. The layout of the microscope is shown in figure 2, and detailed schematics of the SiriusXT patented plasma-based source and SXM beamline are presented in figures 3(a) and (b). The microscope system includes three main modules: the source chamber that generates the soft x-rays, the microscope chamber hosting the x-ray optics, the cryo sample chamber allowing the manipulation of specimens at −180 °C, and the CCD camera. The whole system is positioned on an optical table with a footprint of 2 × 3 m. Soft x-rays are generated by a laser-driven process whereby a 25 mJ, 7 nm pulse at 1 kHz from a standard machining laser generates a hot plasma on a rotating molybdenum (Mo) target. Highly ionized Mo emits strongly in the 2–8 nm spectral region; see figure 3(c). A Cr/Ti multilayer, reflecting at 2.73 nm, filters the broadband source to x-rays in the water window with

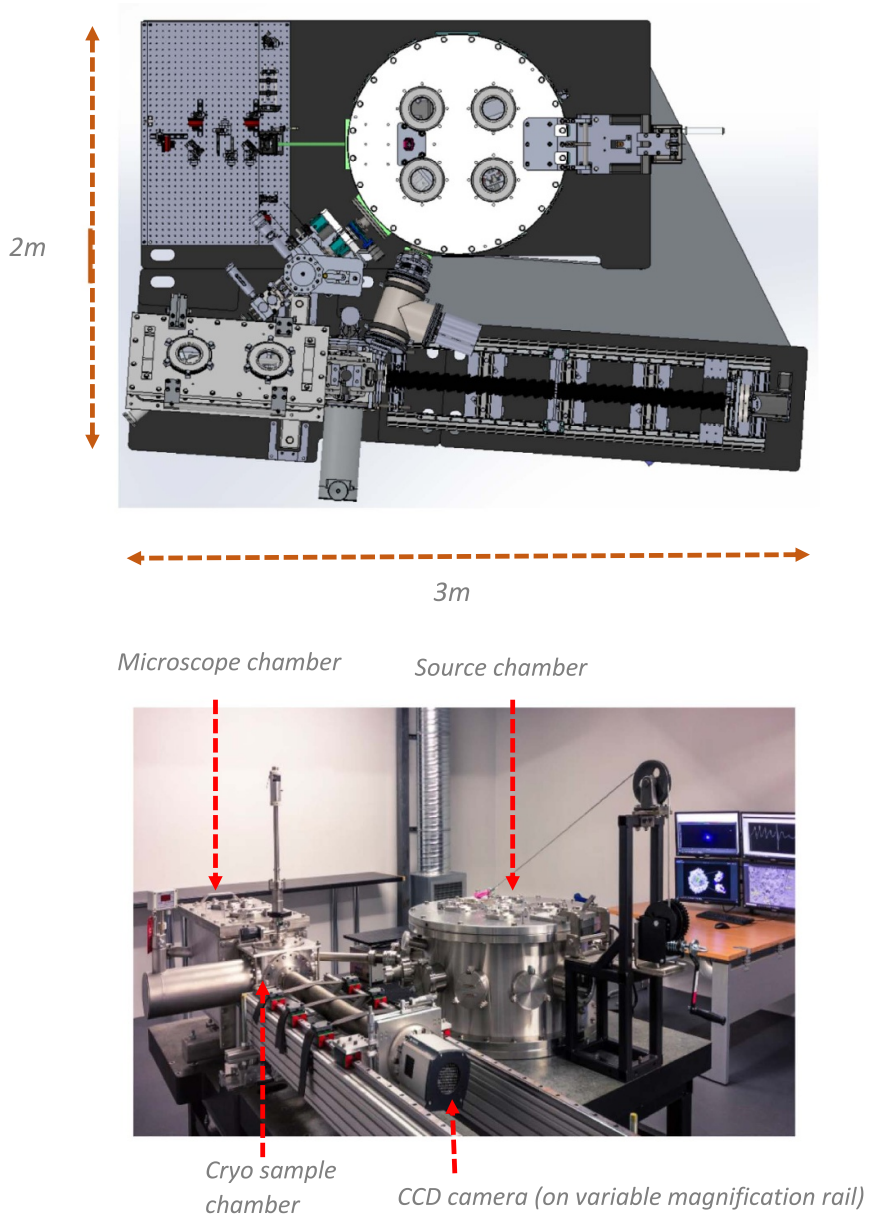

Figure 2. Schematic (top) and image (below) of the prototype lab-scale SXM to illustrate its footprint and main modules. The microscope is modular for ease of assembly and serviceability.

$E/\Delta E \sim 340$ (figure 3(d)). This broad spectral bandwidth is similar to the one reported for SXM at the XM-2 beamline of the ALS [27] and is expected to provide comparable a depth of focus of several micrometres, sufficient to image through whole cells about $\sim$10 μm in diameter. In the sample plane, the illumination spot size provides a $\sim$20 μm field of view, currently with a flux level of $\sim 3 \times 10^5$ photons s^{-1} μm^{-2} (figure 3(e)). The patented technology prevents plasma debris from spoiling nearby laser and x-ray optics, facilitating a stable, bright, and robust table-top soft x-ray beamline.

In the current prototype, a Fresnel zone plate with an outermost zone width of 35 nm is used as the objective lens. To demonstrate the imaging performance of the lab-scale SXM, we imaged 2D resolution test patterns containing nanoscale line spacing at ambient temperatures. A line spacing of 60 nm was consistently resolved at a contrast of 30%, see figure 3(f). To date, a half-pitch resolution of 40 nm with a corresponding depth of field of 6 μm has been achieved using a 35 nm objective zone plate on a Siemens star test sample. The first 2D x-ray projection images of diatoms clearly resolve the overlapping halves and intricate inner markings (figure 3(g)). For the imaging of frozen specimens, a cryo stage and cryo sample-handling tools are integrated and are currently being tested. The next phase of development involves further resolution optimisation (to <30 nm half pitch) as well as 3D tomography on a range of samples, both dry and frozen, to establish the volumetric imaging capability of the lab-scale SXM.

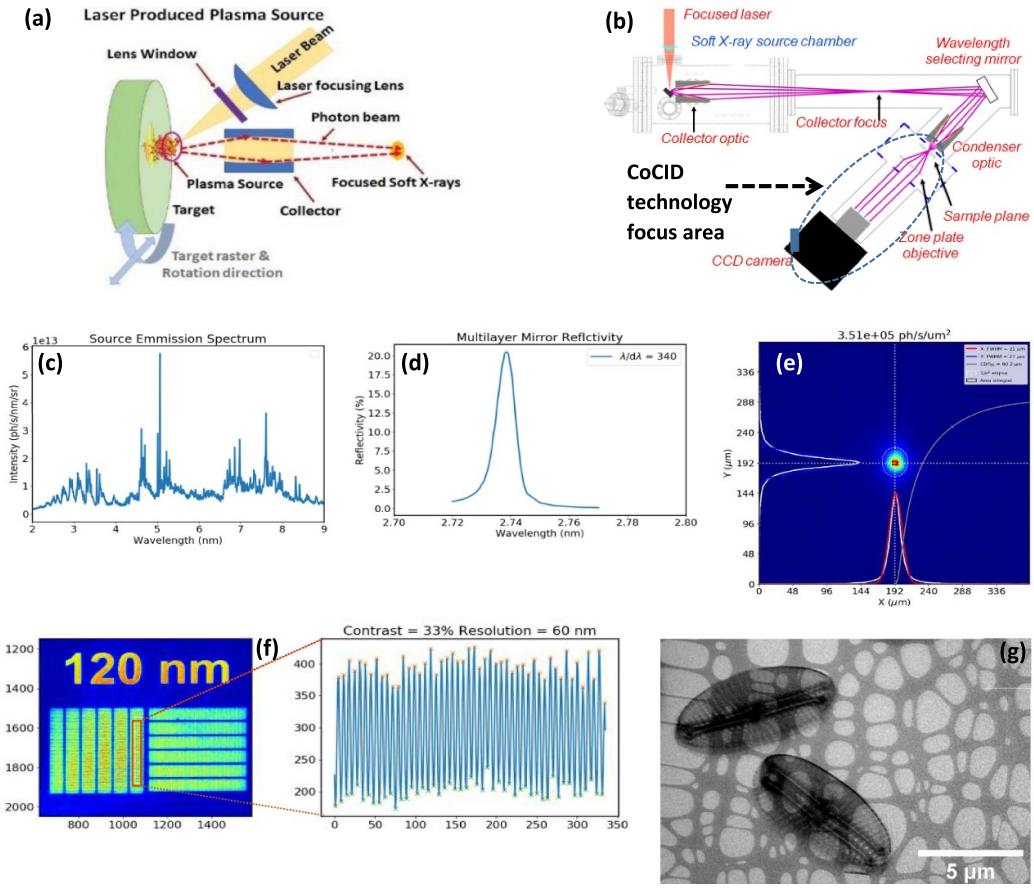

Figure 3. Overview of the lab-scale SXM concept. (a) Patented source technology deals with laser plasma debris allowing higher photon collection efficiencies and robust continuous source operation. (b) Schematic of the lab-scale soft x-ray microscope. (c) Soft x-ray source emission spectrum. (d) Multilayer mirror reflectivity and bandwidth providing quasi-monochromatic sample illumination. (e) Illumination spot size and flux at the sample plane with a $\sim$20 μm field of view with a current flux level of $\sim 3 \times 10^5$ photons s^{-1} μm^{-2}. (f) Image of 60 nm half-pitch vertical and horizontal lines at a contrast level of $\sim$30%. (g) 2D image of diatoms acquired at ambient temperatures.

2.3. Virology use cases

The emerging SXM technique, which possesses high 3D spatial resolution, penetration depth, and sensitivity based on natural x-ray absorption contrast, allows us to address fundamental questions about the cellular origins of viral diseases and the development of associated antiviral and oncolytic therapies.

2.3.1. HCV

Chronic HCV infections induce structural alterations of the mitochondria and endoplasmic reticulum. These changes, caused by active HCV replication, can be reversed by a prolonged treatment with clinically approved direct-acting HCV antivirals, for example, a combination of sofosbuvir and daclatasvir [28]. Our goal is to understand how virus-induced changes in cellular structures are reversed after pharmacological elimination of viral machinery from the host cell. SXM imaging at the ALBA synchrotron [29] has shown that various antiviral drugs lead to differential efficiency in the reversion of the structural alterations in HCV-infected cells [16]. To gain further insights into the mechanisms of cellular processes that govern the recovery of cellular components, we will use lab-scale SXM and advanced data analysis of HCV-replicating cells in the presence of different inhibitors of cellular proteostasis pathways during antiviral treatment.

2.3.2. HEV

Due to the lack of *in vitro* model systems that support robust HEV infection, few studies have attempted to elucidate the infection steps, i.e., viral entry, replication, and assembly. Here, we will use recently developed model systems for HEV, such as pig liver tissue and 3D organoids generated from human cell lines, to recapitulate the uniquely polarized nature of hepatocytes. We will visualize sites of HEV replication and assembly in pig and human polarized and non-polarised cell lines, and confirm our findings using HEV-infected liver slices by lab-scale SXM combined with other imaging techniques, such as electron and

light microscopy. Imaging with SXM will bridge the gap between previously applied microscopy techniques, and will serve as an interface for correlative studies. These correlative studies, in combination with human and pig hepatocyte systems, will allow us to perform a full analysis of the HEV lifecycle and the effect of antiviral therapeutics and inhibitors on HEV infection.

2.3.3. SARS-CoV-2

Currently, little is known about how SARS-CoV-2 exploits cellular resources to achieve high-level virus propagation. Moreover, it is also unclear to what extent disease severity is linked to the direct cytopathogenity of the virus versus an inflammation-dominated immune response. First insights into these questions have mostly been gained using cell lines, but the physiological relevance of the results obtained remains to be determined. Moreover, it is unclear whether host cell perturbations induced by SARS-CoV-2 differ between cell types, which calls for validation studies in physiologically relevant (ideally 3D cell culture) models. This set of questions calls for a multi-scale and correlative imaging approach, from the whole cell to the subcellular, and even molecular levels. We will combine recently established integrative imaging approaches [18, 30] with advanced lab-based SXM and automated segmentation based on machine learning to establish a 3D model for the intracellular modification of SARS-CoV-2 infected cells. Quantitative and spatiotemporally resolved analyses of the virus-induced morphological changes of infected cells will reveal new principles of cellular homeostasis and its perturbation by SARS-CoV-2 infection. These studies will provide important information about viral and cellular targets that might be suitable for the development of antiviral therapies.

2.3.4. HSV-1

Since intranuclear chromatin reorganization is potentially crucial for the nuclear egress of capsids [13, 14] it is essential to understand how the infection induces changes in chromatin. This is important from the perspective of basic research into virus–cell interactions, and also for the improvement of oncolytic HSV-1-mediated virotherapy, which requires effective cellular release and the transduction of newly formed viruses to neighbouring cells [31, 32]. Little is currently known about the mechanisms of virus-induced changes in the chromatin architecture and the mobility of viral capsids in chromatin. Here, we will employ a strategy integrating 3D soft x-ray imaging of chromatin remodelling [13–15], fluorescent microscopy, and advanced data analysis and modelling in order to study the HSV-1-induced changes in the molecular organization of host chromatin in more detail.

3. Discussion

SXM is an ideal nanoscale imaging system which allows high-resolution analysis of single cells at high precision, thus presenting novel insights into the mechanisms of cellular phenomena, such as virus–cell interactions. However, the progress in cell research by SXM has been hampered by the fact that there are only five synchrotron facilities around the world with an SXM, and all of them are heavily oversubscribed. This makes SXM highly inaccessible to the wider scientific community. Moreover, users have to undergo prolonged application procedures for beam time, which might take up to 12 months until permission for the actual experiment, if successfully evaluated, is granted. Once acquired, the limited imaging time might lead to challenges in collecting a statistically significant number of parallel samples and complicated setup logistics for cell sample preparation. CoCID will lead to the first-ever deployment of a commercial laboratory-scale SXM microscope in the worldwide biomedical field. The CoCID consortium will demonstrate the value of using lab-based SXM in four separate research projects/use cases. These studies will demonstrate the benefits of the lab-based system relative to both synchrotron-based SXM and other imaging modalities. The most comprehensive view of complex cellular structures is unlikely to come from a single microscope. Much effort is now invested in combining SXM with other imaging methods, in particular, cryogenic fluorescence microscopy. CoCID will facilitate the correlation of SXM with light and electron microscopy by integrating a fluorescence microscope, and offering a dual-modality sample presentation scheme, including electron microscopy (EM) grids, and an automatic data analysis pipeline. Hybrid imaging with lab-based SXM microscopy and super-resolution fluorescence microscopy enables the correlation of fluorescent data with 3D whole-cell images, at a 20–50 nm resolution. This allows the identification of viral-infection-induced structural changes in the host cell. Based on these findings, the specific regions of interest can be further examined by EM at a 1 nm resolution. Moreover, cryo-correlative workflows could be developed to allow imaging of the same cell using all three modalities: SXM, cryo-EM, and light microscopy. In the first instance, this could be done without any additional sample machining by using cryo-EM to image only the outer ‘thin’ regions of the sample and SXM to image the much thicker cytoplasmic and perinuclear regions.

The lab SXM will be commercially available by the end of the project.

Data availability statement

No new data were created or analysed in this study.

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