

Correlative X-ray imaging and fluorescence microscopy in hydrated environments

Mangalika Sinha,^{a,b,†} Boram Yu,^{a,†} Rita Mendes Da Silva,^{a,c} Ulrike Rölleke,^a Peter Luley,^a Malte Tiburcy,^{d,e} Wolfram-Hubertus Zimmermann,^{b,d,e} Manfred Burghammer^c and Sarah Köster^{a,b,*}

Received 28 June 2026

Accepted 10 September 2026

Edited by M. Guizar-Sicairos, Paul Scherrer Institute, Switzerland, and EPFL, Switzerland

† These authors contributed equally to this work.

Keywords: correlative imaging; fast scanning SAXS; fluorescence microscopy; biological cells; microfluidics.

Supporting information: this article has supporting information at journals.iucr.org/s

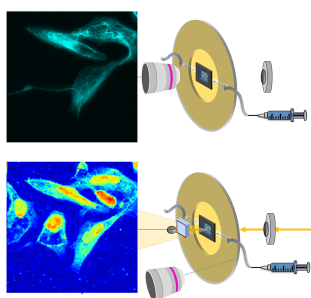
^aInstitute for X-ray Physics, University of Göttingen, Friedrich-Hund-Platz 1, 37077 Göttingen, Germany, ^bCluster of Excellence 'Multiscale Bioimaging: From Molecular Machines to Networks of Excitable Cells' (MBExC), 37075 Göttingen, Germany, ^cESRF – The European Synchrotron, 71 Avenue des Martyrs, 38043 Grenoble Cedex 9, France, ^dInstitute of Pharmacology and Toxicology, University Medical Center Göttingen, 37075 Göttingen, Germany, and ^eGerman Center for Cardiovascular Research (DZHK), 37075 Göttingen, Germany.

*Correspondence e-mail: sarah.koester@uni-goettingen.de

Imaging cellular organization in its native state is essential for understanding how the molecular architecture governs biological function. Fluorescence microscopy is one of the pivotal techniques that provides molecular specificity for imaging in real space, but is limited to labeled components. In contrast, X-rays are sensitive to electron density variations, enabling label-free structural characterization in reciprocal space. In particular, scanning small-angle X-ray scattering (SAXS) combines information from real and reciprocal space and enables access to intact cells owing to the high penetration power of X-rays. To harness the complementary strengths of these approaches, we developed a correlative imaging platform integrating a modular, compact, and beamline-compatible fluorescence microscope with scanning SAXS. The system enables fast sequential imaging of the same cellular regions under hydrated, near-native conditions using a dedicated microfluidic chamber. Applying this approach to keratin bundles in epithelial cells and force-generating actomyosin in cardiomyocytes, we demonstrate good agreement between fluorescence and SAXS-derived orientation maps. This result shows that label-free SAXS captures cytoskeletal organization throughout the cell, and can be directly linked to specific molecular information under physiologically relevant conditions. Our work establishes a general strategy for multimodal imaging of cellular architecture and enables studies of living cells under drugs and chemical manipulation.

1. Introduction

Biological function is to a great extent determined by the structural and dynamic properties of the cellular components, including the cytoskeleton, membranes and organelles, such as the nucleus. For this reason, imaging of intracellular structures at high spatial resolution has become an invaluable tool in the life sciences. In particular, correlative imaging that combines two or more different methods reveals complementary information about the system under investigation. Here we combine visible light fluorescence microscopy and X-ray scattering to collect protein-specific structural data from biological cells. In fluorescence microscopy, targeted labeling of specific proteins of interest offers the possibility to selectively visualize these components inside cells. X-ray scattering, by contrast, is a label-free technique, which records scattered photons from all matter within the beam and thereby images electron density contrast directly. X-rays have a small wavelength, enabling high resolution, and a high penetration



power. This property allows us to image ‘thick’ samples like whole cells.

Among the various X-ray imaging techniques for biological cells and tissues that have been developed in recent decades (Hémonnot & Köster, 2017; Albers *et al.*, 2024; Fantoni *et al.*, 2025), scanning small-angle X-ray scattering (SAXS) employs a focused X-ray beam being scanned with respect to the sample plane; a spatially resolved scattering signal is thus recorded from each scan position. Therefore, scanning SAXS in an elegant manner combines real space imaging and reciprocal space structural information. When employing beams with diameters of tens to hundreds of nanometers, they are of the same order as the size of the scatterers, *i.e.* the intracellular components. Therefore, depending on the sample organization and scattering contrast, information on structural parameters such as characteristic length scales, anisotropy, and local orientation may be derived (Priebe *et al.*, 2014; Hémonnot *et al.*, 2016a). Moreover, the anisotropic intracellular structures can give rise to azimuthally dependent scattering, allowing their predominant local orientation to be determined (Weinhausen *et al.*, 2012). Alternatively, the scattering intensities may be analyzed in dependence of the scattering angle q (Weinhausen *et al.*, 2014; Reichardt *et al.*, 2020; Javor *et al.*, 2021; Bruns *et al.*, 2024). Here, we specifically focus on the correlative analysis of fluorescence microscopy and scanning SAXS, with particular emphasis on the spatially resolved scattering intensity providing real-space images and local orientation. When first developed, scanning SAXS has been applied to comparatively radiation-resistant biomaterials such as wood (Lichtenegger *et al.*, 1999), bone (Fratzl *et al.*, 1997; Rinnerthaler *et al.*, 1999; Gourrier *et al.*, 2010), teeth (Tesch *et al.*, 2001; Gaiser *et al.*, 2012), and tendons (Fratzl *et al.*, 2003). More recently, structures in freeze-dried biological cells (Bernhardt *et al.*, 2017) including keratin bundles (Weinhausen *et al.*, 2012; Hémonnot *et al.*, 2016a), actin stereocilia (Piazza *et al.*, 2014), and the DNA in the cell nucleus (Hémonnot *et al.*, 2016b) have been studied. The advantage of studying dry samples lies in the improved electron density contrast between the surrounding air and the cellular material, as well as increased sample stability during the measurements. It is therefore relatively straightforward to reveal quantitative information on structural parameters such as bundle and filament orientation and alignment (Weinhausen *et al.*, 2012) and filament-packing geometries within a bundle (Hémonnot *et al.*, 2016a). However, fixed-hydrated or even living samples are of great interest as they come much closer to the native situation of a biological cell (Weinhausen *et al.*, 2014; Reichardt *et al.*, 2020). Beside the diminished electron density contrast, the increased sensitivity to radiation damage of such samples poses great challenges.

To provide such a near-native, close-to-physiological environment, dedicated microfluidic chambers have been developed and applied (Weinhausen & Köster, 2013; Yu *et al.*, 2026; Köster & Pfohl, 2012; Ghazal *et al.*, 2016). Such flow chambers provide a number of advantages: as the cells are constantly provided with nutrients, waste is transported off, the sample is slightly cooled by the liquid flow, and, impor-

tantly, the generation of gas bubbles by the radiation that would prevent further measurements is greatly diminished. Using such chambers and thereby enabling long total experiment times, recently, fast continuous scanning of large sample areas and, therefore, high numbers of cells was achieved (Cassini *et al.*, 2020; Yu *et al.*, 2026). While microfluidic chambers for sole microscopy applications fabricated by soft lithography (Xia & Whitesides, 1998) are a standard tool in many labs now, the fabrication of a sample chamber that is equally well suitable for microscopy and for X-ray imaging remains challenging. This challenge was also identified in a recent study that applied the combination of X-ray imaging and correlative super-resolution fluorescence microscopy to biological cell samples (Bernhardt *et al.*, 2018; Bernhardt *et al.*, 2019): due to the lack of compatible sample chambers, the cells were studied in a freeze-dried state. Nonetheless, the study impressively showed the great advantage of combining different imaging modalities in a complementary manner.

The eukaryotic cytoskeleton is an important determinant for the mechanical properties of cells. It consists of protein filaments—actin filaments, microtubules and intermediate filaments—that may form functionally relevant ordered structures such as bundles and networks. The cytoskeleton provides suitable test systems for correlative measurements because the proteins can be fluorescently tagged and specifically visualized and the ordered protein structures can generate anisotropic X-ray scattering. Here, we consider two cellular systems with distinct cytoskeletal architectures. First, SW-13 epithelial cells contain prominent bundles of keratin K8/K18 intermediate filaments (Weinhausen *et al.*, 2012; Weinhausen *et al.*, 2014; Hémonnot *et al.*, 2016a). Second, we investigate human induced pluripotent stem cell (hiPSC)-derived cardiomyocytes, which contain organized actomyosin structures (Reichardt *et al.*, 2020; Javor *et al.*, 2021). Notably, in the hiPSC-derived cardiomyocytes, the actomyosin organization is spatially heterogeneous and the Z-disks are less mature and less continuously organized than in adult cardiomyocytes. We consider this system the more challenging sample than the SW-13 cells for the correlative approach due to weaker and more heterogeneous local scattering contrast.

Here, we present the design, construction and application of a compact, high-quality fluorescence microscope that is integrated in a synchrotron beamline in a straightforward manner. We combine the microscope with a dedicated sample environment, *i.e.* a microfluidic chamber that fulfills all necessary requirements: it is compatible with scanning SAXS and transparent to X-rays, it is compatible with fluorescence microscopy and moderate working distance objectives, it includes a thin water layer only, thus providing good X-ray contrast, and it enables continuous flow to diminish X-radiation damage and gas bubble formation. We apply the novel development to the two cellular systems mentioned above: first, pronounced keratin bundles in SW-13 cells and, second, force generating actomyosin in hiPSC-derived cardiomyocytes. Our approach enables a smooth workflow that was previously missing, including a compact design suitable for various beamlines, fast switching between the imaging

modalities, the possibility for extended investigation of biological cells in a near-native, aqueous environment and the applicability to different (biological and non-biological) sample types. In this work, we focus on quantifying ordered structures in both fluorescence microscopy and scanning SAXS data, and the orientation maps derived from both modalities show great agreement. Thus, our setup demonstrates the benefit of multimodal imaging of biological systems.

2. Results

2.1. Integrated microscopy setup and microfluidics device

Combined X-ray imaging and visible light fluorescence microscopy promises to provide highly complementary information from biological samples; while scanning SAXS probes structural parameters like alignment and orientation of subcellular components, in a spatially resolved manner, fluorescence microscopy highlights the specifically labeled components and locates them within the context of the cell. To enable correlative imaging with both modalities, we develop an integrated platform combining a compact epi-fluorescence microscope with scanning SAXS and a dedicated microfluidic chamber for measurements under fully hydrated conditions. The home-built compact epi-fluorescence microscope is specifically designed for integration into a synchrotron beamline. Fig. 1(a) shows a schematic of the optical path of the microscope, which comprises a light source and light guide, an aspheric collimation lens, an excitation filter, a dichroic mirror, a high numerical aperture (NA) objective, an emission filter, a tube lens and a sensitive camera, arranged to minimize footprint while maintaining excellent optical performance. Here, a GFP filter set is used; however, it can be changed to filter sets of different wavelengths in a straightforward manner. The specifications of all the optical components used in the set-up are provided in Table S1 of the supporting information. We employ a 20 $\times$, NA 0.7 objective with a working distance of 1 mm, as an optimal compromise between sufficiently high lateral resolution, a large field of view, and geometrical compatibility with the microfluidic chamber in the beamline set-up. Typically, higher-magnification objectives have much shorter working distances on the order of 0.1–0.3 mm, which are incompatible with the microfluidic chamber geometry. Specifically, we use fragile Si₃N₄ membranes of 1 μ m thickness of the window material as it is compatible with SAXS, microscopy and cell culture. This prohibits the use of immersion objectives that require close proximity to the substrate and risk damaging the membrane. The microscope is mounted on a motorized linear stage that translates the optical axis in and out of the X-ray path with a reproducibility down to 1 μ m, enabling fluorescence imaging without perturbing the specimen position relative to the nanofocused X-ray beam, see Fig. S1 in the supporting information. Fig. 1(b) shows a photograph of the microscope within the X-ray path and in relation to the sample chamber. The main components are colored and explained in the legend.

To provide a stable and fully hydrated environment for both imaging modalities, we develop a dedicated microfluidic chamber. Fig. 1(c) shows a cross-sectional view of the fully assembled microfluidic chamber. The chamber consists of a Kapton–NOA81–Kapton device defining a 1.5 mm-wide microfluidic channel, with 25 μ m channel height. The central observation region is sealed from both sides by Si₃N₄ windows, thereby establishing an observation region that is compatible with both fast scanning SAXS and fluorescence microscopy. Fabrication details are provided in Section 4.2, with a step-by-step overview shown in Fig. S2 of the supporting information. The channel dimensions [see Fig. S3(a) of the supporting information] are optimized to achieve sufficient volume flow rates at the observation site. This prevents sample drying and bubble formation caused by X-radiation. The channel dimensions also ensure the mechanical stability of the flow chamber and laminar flow conditions, as illustrated in the simulation, see Fig. S4 of the supporting information. For additional mechanical stability, the device is mounted on a polydimethylsiloxane (PDMS) support containing a 3 cm-diameter opening. This provides unobstructed access for a 20 $\times$, NA 0.7 objective. The enclosed water layer between the two Si₃N₄ membranes has a total thickness of approximately 60 ± 10 μ m. This reduced water path length substantially decreases absorption, thereby improves the X-ray scattering contrast and enables exposure times as short as 2 ms.

The complete integration of the fluorescence microscope, microfluidic chamber, and scanning SAXS geometry is illustrated in Fig. 1(d). The microfluidic chamber is mounted on a scanning stage consisting of a hexapod for coarse positioning and a piezo stage for fine translations during both the microscopy and the SAXS measurements. During microscopy, the home-built epi-fluorescence microscope is moved along the y-axis and positioned in the X-ray path, thus aligning it with the observation region (*i.e.* the cells in the microfluidic chamber) [Fig. 1(d)(i)]. During SAXS acquisition, the microscope is retracted along the y-axis, and the X-rays are focused on the cells in the microfluidic chamber, see Fig. 1(d)(ii). Importantly, this geometry avoids repositioning the chamber itself, enabling rapid alternation between the home-built microscope and scanning SAXS measurements and facilitating spatial registration between the two modalities. Further details of the integrated setup are provided in Section 4.4.

Prior to the Extremely Brilliant Source (EBS) upgrade at The European Synchrotron (ESRF), we performed measurements on fixed-hydrated SW-13 cells using a microfluidic chamber with a water layer of 200 μ m, lower photon flux, and longer exposure times (Weinhausen, 2013). A comparison of the corresponding dark field images is shown in Fig. S5 of the supporting information and the experimental parameters before and after the EBS upgrade are also summarized in Table S2 of the supporting information. Despite considerably shorter exposure times, we observe that the maximum dark-field contrast increases by nearly two orders of magnitude due to the higher photon flux. In addition, the thinner water layer enhances the scattering contrast, defined as $(I_{\max} - I_{\min})/I_{\min}$, by approximately a factor of two. Together, the reduced

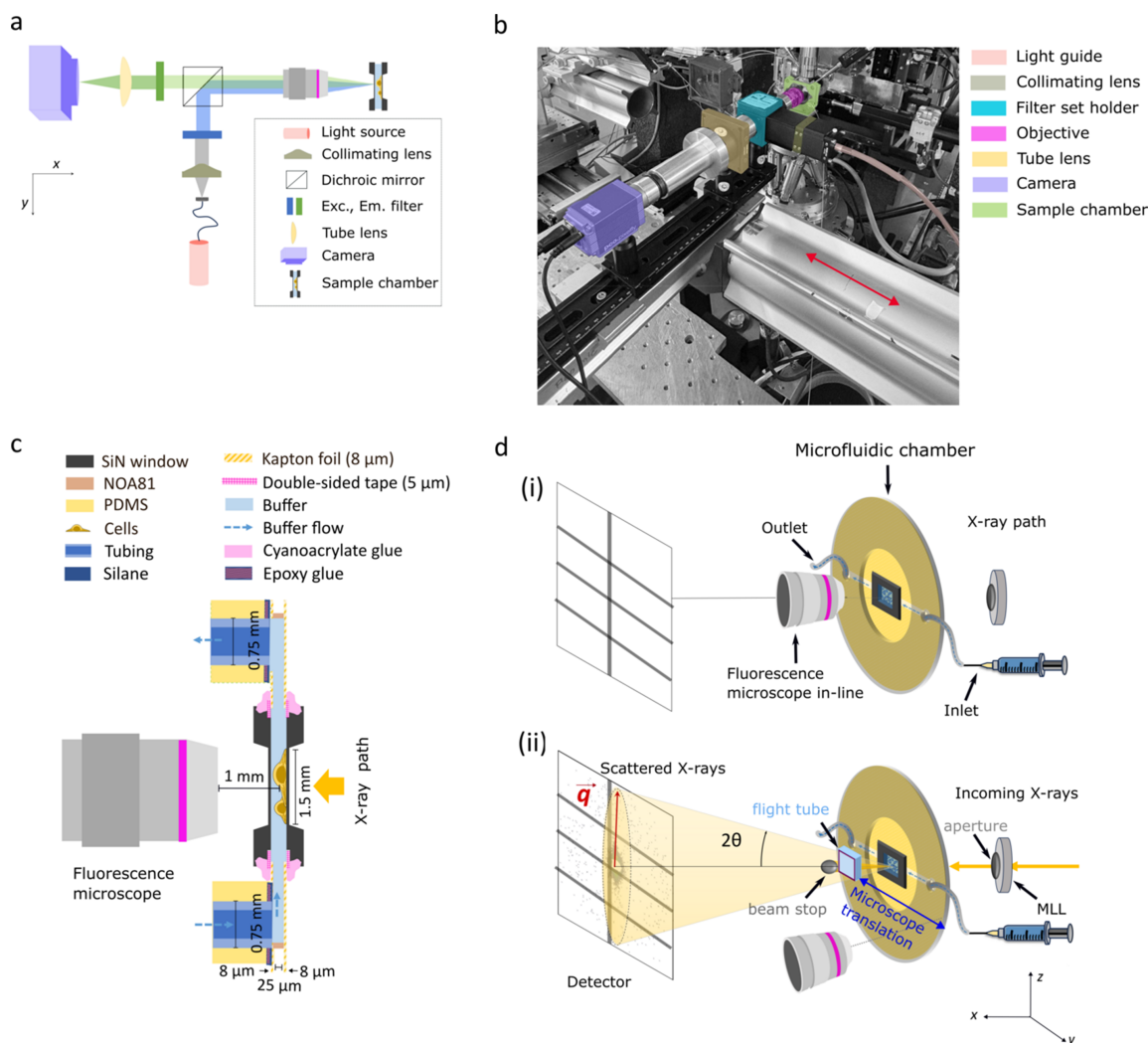


Figure 1

Integrated fluorescence microscopy and microfluidic sample environment for multi-modal correlative imaging at a synchrotron beamline. (a) Schematic of the home-built epi-fluorescence microscope showing the optical layout optimized for integration in the beamline. (b) Photograph of the microscope installed at the beamline (ESRF, ID13). The relevant optical components are highlighted in different colors. In this geometry the sample stays in the X-ray beam path; fluorescence imaging is performed sequentially to the X-ray measurements by shuttling the microscope in and out of the X-ray beam path, as indicated by the red arrow. (c) Side-view schematic of the fully assembled microfluidic chamber and the geometry of the setup with the fluorescence microscope in-line while imaging. See legend for the components of the flow chamber and Fig. S2 of the supporting information for a step-by-step explanation of the assembly. (d) Schematic of the integrated experimental setup (not to scale). (i) Microscopy mode. The home-built fluorescence microscope aligned with the microfluidic chamber for imaging. In this mode, no incoming X-ray beam is present. (ii) Fast scanning SAXS mode. The microscope is moved out of the X-ray path to enable fast scanning SAXS measurements. The incoming X-rays are focused by multilayer Laue lenses (MLLs), followed by an aperture placed downstream the MLLs, before illuminating the cells present in the microfluidic chamber. The primary beam is blocked by a beam stop and the scattered signal is collected by the detector. The red arrow illustrates the direction of the corresponding scattering vector $\mathbf{q}$ for a scattering angle 2θ . The biological cells are maintained in a thin hydration layer between two Si_3N_4 windows integrated in the microfluidic chamber, including an inlet (connected to a syringe) and an outlet for continuous buffer flow.

chamber thickness and higher photon flux enable rapid measurements with substantially enhanced scattering contrast without increasing the radiation dose. To maintain hydration and suppress X-ray-induced gas bubble formation, buffer is continuously perfused through the chamber during measurements. The experiments are typically conducted at a flow rate of $100 \mu\text{L h}^{-1}$. A summary of flow rates and corresponding velocities is provided in Table S3 of the supporting information. The chamber operates stably over a range of 20–200 $\mu\text{L h}^{-1}$.

Taken together, the compact fluorescence microscope, reduced water layer microfluidic chamber, and retractable

microscope geometry provide an integrated platform for correlative fluorescence microscopy and fast scanning SAXS under hydrated conditions. This setup enables precise correlative fluorescence and X-ray imaging with minimized time delay in between.

2.2. Characterization of the fluorescence microscope setup

To assess the quality of the imaging achieved by our home-built fluorescence microscope, we benchmark it against a commercial research microscope (IX83, Olympus, Hamburg, Germany) equipped with the same objective and filter set. We

determine the lateral point spread function (PSF) by preparing samples of 0.1 μm -diameter yellow-green fluorescent beads dissolved in ultrapure water at a final concentration of 0.1 $\mu\text{g ml}^{-1}$, on plasma-activated Si_3N_4 membrane windows. The beads are allowed to adhere during a one hour incubation period. After incubation, the bead-coated window is integrated within the microfluidic chamber specifically designed for this study and the beads in aqueous environment are imaged. Images of individual beads are analyzed by extracting horizontal and vertical intensity profiles through the centroids. The intensity profiles from $N = 14$ individual beads are first smoothed, normalized, aligned, linearly interpolated and subsequently averaged to obtain representative average bead profiles for the horizontal (H) and vertical (V) directions. Gaussian functions are then fitted to the averaged bead profiles and the lateral PSF is determined as the full width at half-maximum (FWHM), see Fig. 2(a). The home-

built microscope yields results for the FWHM of $0.87 \pm 0.11 \mu\text{m}$ (H) and $0.85 \pm 0.14 \mu\text{m}$ (V), indicating a sub-micrometer PSF with minimal astigmatism, see Fig. 2(a)(i,ii). Measurements with the commercial microscope yield mean FWHM values of $1.00 \pm 0.13 \mu\text{m}$ (horizontal) and $0.99 \pm 0.15 \mu\text{m}$ (vertical), see Fig. 2(a)(iii,iv). The small deviation of $\sim 0.1 \mu\text{m}$ in the FWHM between the two systems lies within the pixel-sampling uncertainty and does not reflect a true optical performance difference, as the effective pixel sizes at the specimen plane are nearly identical, *i.e.* 0.323 μm per pixel for the home-built microscope and 0.325 μm per pixel for the commercial microscope. Fig. 2(b) summarizes the bead-by-bead analysis of the FWHM values as box plots. Example bead images and their corresponding horizontal and vertical line intensity profiles for both the home-built and commercial microscopes are shown in Fig. S6 of the supporting information. This bead-by-bead analysis captures the position-

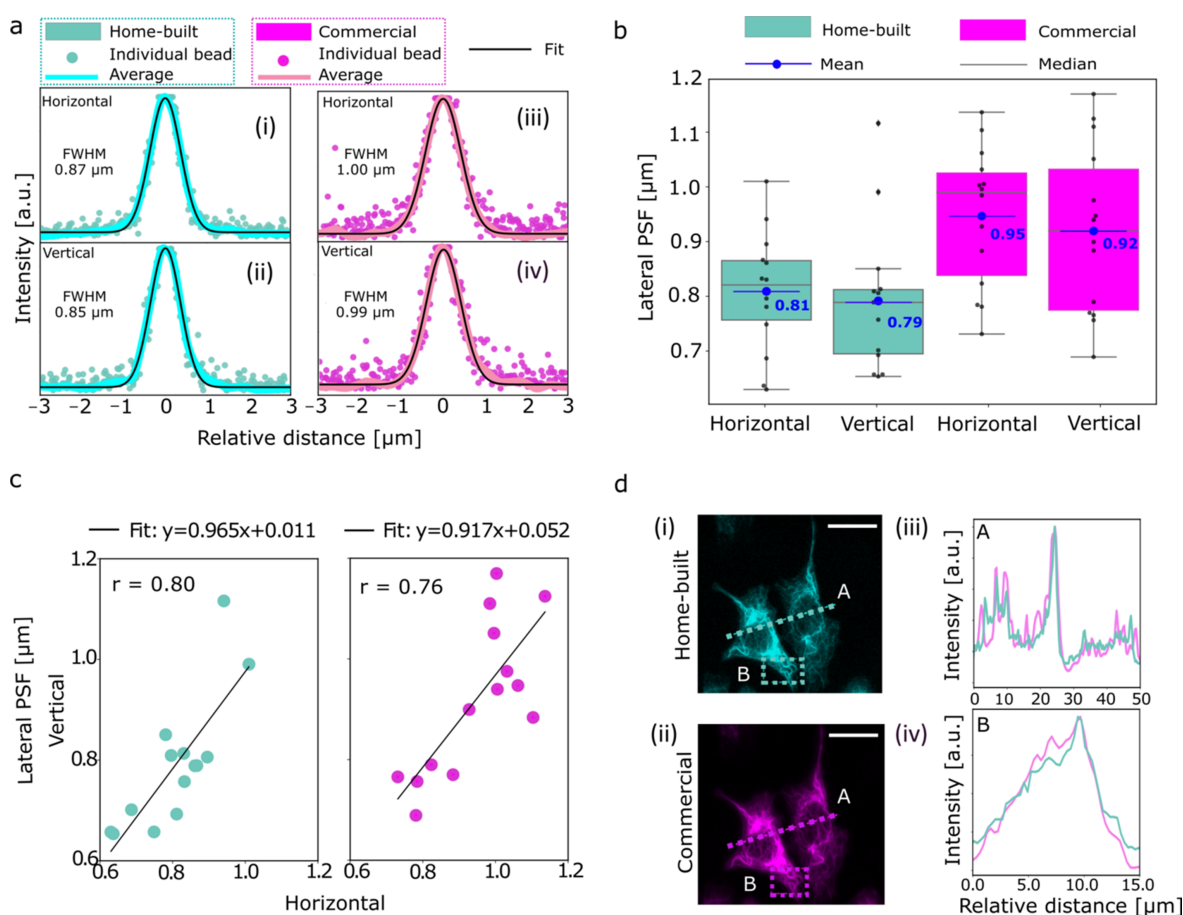


Figure 2

Performance characterization and validation of a compact fluorescence microscope for use in a synchrotron beamline. (a) Evaluation of the lateral PSF of the microscope using 0.1 μm -diameter fluorescent beads suspended in ultrapure water and deposited between two Si_3N_4 windows arranged in analogy to the actual measurements. Panels (i) and (ii) show the individual horizontal and vertical line intensity profiles of $N = 14$ beads (cyan, scatter) along with the averaged intensity profile (cyan, solid line) and the corresponding Gaussian fit (black, solid line). Panels (iii) and (iv) show the corresponding measurements for a commercial epi-fluorescence microscope (shown in magenta). Identical objectives and filter sets are used in both configurations. The FWHM denotes the lateral point spread function of the averaged horizontal and vertical profiles. (b) Quantitative comparison of the lateral point spread function between the home-built beamline microscope and the commercial system. The FWHM from the Gaussian fits of the individual horizontal and vertical line profiles of each of the $N = 14$ beads for each case are plotted as single data points (black). The box plots indicate the range of values between the 25th percentile and 75th percentile with the median (gray) and the mean (blue) of the distributions. (c) Correlation of bead FWHM in the horizontal and vertical axes in the focal plane, illustrating the degree of astigmatism. Each point represents an individual bead; the black lines show linear fits. (d) Images of keratin bundles in cells acquired from the same region on a sample using the home-built and a commercial microscope (scale bars: 20 μm). The normalized intensity profiles for A (dotted line) and B (dotted box) agree well.

dependent measurement variability across the field of view. We exclude aggregated beads based on their visual appearance. The measured characteristics of individual beads vary with their location in the Si_3N_4 membrane and this effect is not accessible from averaged profiles alone.

In an ideal, aberration-free imaging system, the lateral PSF is expected to be isotropic, yielding identical horizontal and vertical FWHM values for each bead, and, consequently, a perfect linear correlation with $r = 1.0$. Deviations from this ideal case arise from residual optical imperfections such as weak astigmatism, alignment tolerances, or sampling effects introduced by the camera pixel size. Correlation analysis between the horizontal and vertical FWHM values of the beads shows a strong linearity in both microscopes of $r = 0.80$ for the home-built microscope and $r = 0.76$ for the commercial microscope, see Fig. 2(c). This high degree of correlation indicates that the lateral resolution in both systems is not dominated by axis-specific aberrations.

Finally, we validate the performance of the home-built microscope on a biological specimen, *i.e.* fluorescently labeled keratin bundles in fixed cells. Representative images acquired using the home-built (cyan) and commercial (magenta) microscopes include a line scan (A, dotted line) and a rectangular region of interest (ROI) (B, dotted box) for comparison, see Fig. 2(d)(i,ii). The normalized intensity profiles extracted along the line scan provide qualitative visualization of variation in fluorescence intensity along this line, see Fig. 2(d)(iii). However, since a line scan takes into account only limited number of pixels, the resulting profile is sensitive to shot noise, camera noise and precise placement of the line scan. For a further comparison of the two microscopes, we therefore extract normalized averaged intensity profiles from rectangular ROIs, see Fig. 2(d)(iv). The signal-to-noise ratio (SNR) and the contrast-to-noise ratio (CNR) are calculated from background-subtracted intensity profiles (B, dotted box region) using automatically detected signal peaks. The background level and noise are estimated from the lowest 20% intensity values of the profile. For each detected peak, the mean signal intensity μ_{signal} is determined from a local region surrounding the peak (± 3 pixels around the peak maximum). The SNR is then calculated as $\text{SNR} = \mu_{\text{signal}} / \sigma_{\text{background}}$, where $\sigma_{\text{background}}$ is the standard deviation of the background intensity. The CNR is calculated as $\text{CNR} = (\mu_{\text{signal}} - \mu_{\text{background}}) / \sigma_{\text{background}}$, where $\mu_{\text{background}}$ denotes the mean background intensity. The final SNR and CNR values are obtained by averaging over all detected peaks within the ROI. The home-built microscope exhibits an SNR of 21.2 compared with 15.5 for the commercial microscope, while the CNR is 11.15 for the home-built system and 9.42 for the commercial system.

This benchmarking shows that our compact home-built fluorescence microscope for integration into a synchrotron beamline achieves sub-micrometer optical resolution and an imaging performance comparable with state-of-the-art commercial research microscopes. At the same time, it satisfies the stringent mechanical, steric and operational constraints imposed by the synchrotron beamline environment.

2.3. *In situ* immunostaining

The controlled perfusion capability of the microfluidic chamber enables *in situ* delivery of reagents to the cells inside the chamber. To demonstrate this capability, we perform immunostaining of intracellular keratin networks inside the chamber. For this proof-of-principle experiment, fixed-hydrated SW-13 cells stably expressing fluorescent keratin are used, providing a reference for the quality of the immunostaining. Cells grown on Si_3N_4 membranes are integrated into the microfluidic chamber, followed by imaging the keratin structures as shown in Fig. 3(a, cyan). This is followed by immunostaining including sequential perfusion steps as detailed in Section 4.3. Following the staining, keratin structures are imaged again as shown in Fig. 3(b, magenta) and agree with the control image shown in Fig. 3(c, overlay).

Taken together, these results demonstrate the benefit of the microfluidic chamber for controlled *in situ* delivery of reagents to cells while maintaining the sample in a hydrated environment. The successful immunostaining of the keratin networks shows that sequential perfusion steps can be performed directly inside the chamber without compromising the structural integrity of the cellular network. This capability provides a foundation for experiments on living cells related to drug addition or labeling. The platform is broadly applicable across cell types, staining strategies, and buffer conditions, and is compatible with both fixed and living cells.

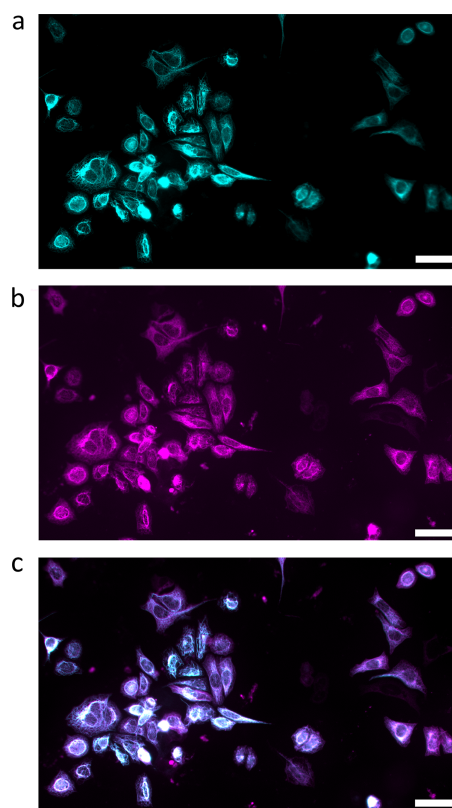


Figure 3 Fluorescence images of fixed-hydrated keratin-rich SW-13 cells before (a, cyan) and after (b, magenta) *in situ* immunostaining inside the assembled device. The overlay (c) of the keratin structures before and after the *in situ* immunostaining shows good agreement. Scale bars: 50 μm .

2.4. Multi-modal imaging of biological cells

The compact fluorescence microscope for beamline integration in combination with the dedicated microfluidic chamber enables sequential microscopy and fast scanning SAXS measurements of identical cellular regions under hydrated conditions. To demonstrate the versatility of this workflow, we apply the approach to two cell types with distinct cytoskeletal architectures: keratin bundles in SW-13 epithelial cells and force-generating actomyosin in hiPSC-derived cardiomyocytes.

2.4.1. Keratin bundles in SW-13 epithelial cells

SW-13 epithelial cells provide an ideal model system to investigate cytoskeletal organization due to their prominent and strongly bundled keratin K8/K18 intermediate filament network, which dominates the intracellular architecture. These cells stably express fluorescent keratin fusion proteins (HK8-CFP and HK18-YFP), enabling direct visualization of keratin organization without immunostaining. Fluorescence imaging reveals pronounced spatial heterogeneity within the keratin network, see Fig. 4(a). The inverted fluorescence

image shows wavy filament structures (black arrow) as well as straightened, highly ordered keratin bundles (black box) along with regions of dense keratin networks (red arrow). The corresponding X-ray dark-field image of the selected scan region is shown in Fig. 4(b). The contrast between the cytoplasm (green) and nuclei (red) is well visible, indicating a good scattering contrast. This is achieved by integrating the scattering intensity over an optimized q -range, as described in Section 4.5.1 (Yu *et al.*, 2026). Notably, regions with dense keratin networks, as indicated by the red arrow in Fig. 4(a), exhibit a higher scattering intensity, see Fig. 4(b), than those with lower keratin density.

In addition to the cells visible in the fluorescence image, the X-ray dark-field image reveals additional cells that do not express keratin and therefore lack fluorescence signal. This highlights the advantage of SAXS, where image contrast arises from electron density differences, independent of fluorescent labeling, enabling visualization of both labeled and unlabeled cellular components. Fig. 4(c) shows the keratin filament network detected from the fluorescence image using the software package *SOAX* (version 3.7.0) (Xu *et al.*, 2015), with filaments color-coded according to their local orientation. The

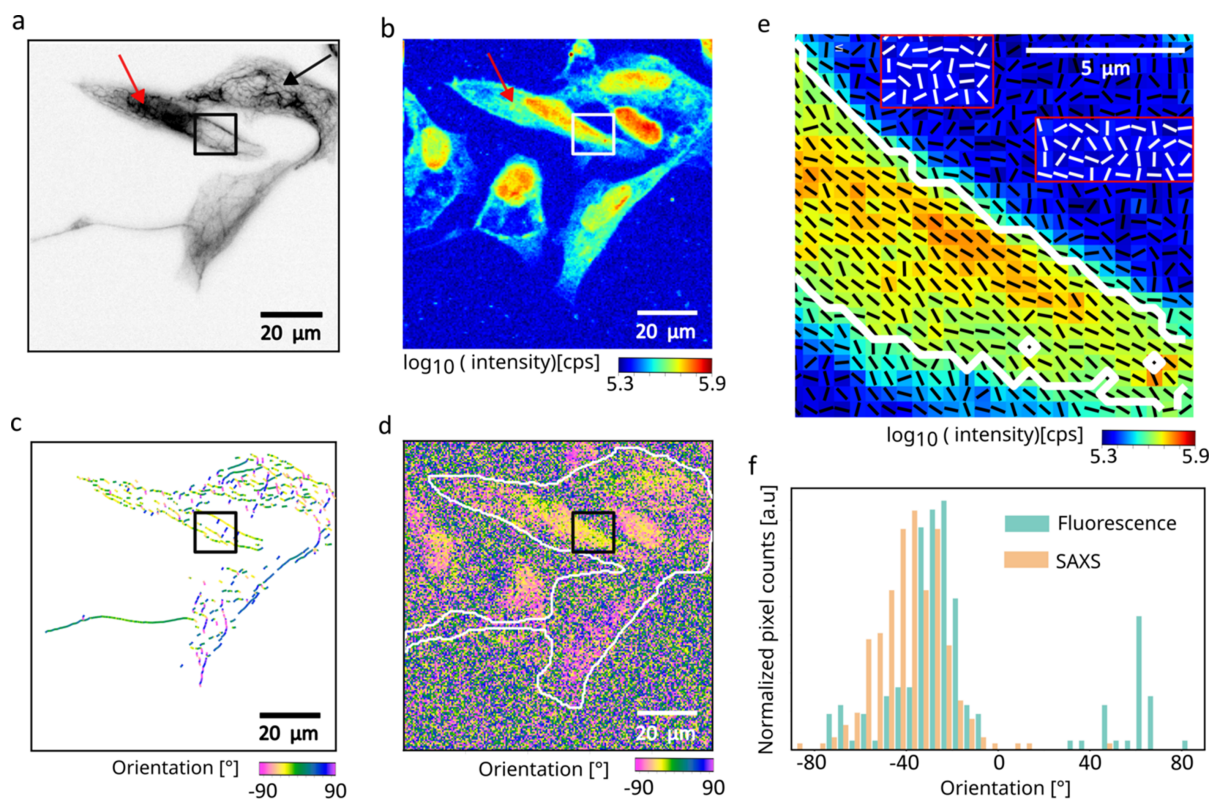


Figure 4

Correlative fluorescence and fast scanning SAXS analysis of keratin filament organization in SW-13 cells. (a) Inverted fluorescence image of keratin-rich SW-13 cells acquired using the home-built fluorescence microscope at the beamline. The black box highlights an area containing ordered keratin bundles, the black arrow points to wavy keratin structures, while the red arrow points to a dense keratin network. (b) X-ray dark-field image obtained from the scanning SAXS corresponding to the same region as shown in panel (a). The X-ray dark-field image reveals additional cells that do not express keratin and therefore do not show a fluorescence signal. (c) Bundle networks are detected from the fluorescence image using *SOAX* and are color-coded by their orientation. (d) SAXS-derived orientation map. The white outlines indicate the keratin-expressing cells as shown in panel (a). (e) Overlay of the X-ray dark-field image with black lines representing the SAXS-determined orientation of ordered structures as shown in the black box in panel (a). Selected background regions are marked by red boxes, with white line segments indicating their corresponding orientations for enhanced visibility. Pixels within the white contour correspond to areas of high X-ray scattering intensity within the ordered structure. (f) Orientation histograms obtained from SAXS and fluorescence analysis for the same region within the white outline in panel (d) show consistent alignment trends between the two modalities.

region containing highly ordered keratin bundles [black box in panel (a)], exhibits a consistent orientation, indicated by the yellow color corresponding to $\sim -30^\circ$.

Fig. 4(d) shows a scanning SAXS-derived orientation map, revealing local variations in filament alignment (Yu *et al.*, 2026). An orientation of 0° corresponds to the horizontal direction [Figs. 4(c) and 4(d)]. Regions containing ordered keratin bundles exhibit more uniform filament orientations, reflected by similar color values. Interestingly, both the orientation maps from fluorescence microscopy and from scanning SAXS show the same orientation distributions, see, for example, the yellow color in the black box region. This result shows that both methods consistently detect oriented structures in the cells. Unlike fluorescence microscopy, SAXS is not specific to a particular labeled component and reports on the dominant orientational order arising from all anisotropic structures within the scanned region of the cell. A close inspection of the region highlighted by the black box shown in Figs. 4(c) and 4(d) reveals agreement between the orientations obtained from fluorescence microscopy and SAXS, indicated by similar colors in both plots. This correspondence indicates that the keratin filament bundles considerably contribute to the SAXS-derived orientation.

For improved visualization, Fig. 4(e) shows an overlay of the X-ray dark-field image with black line segments for every scan pixel, representing the SAXS-derived local orientations. Here, the directions of the line segments indicate the directions of the cellular structures in real space and the lengths of the line segments correspond to the degree of anisotropy (Weinhausen *et al.*, 2012). Some of the selected background regions are additionally marked by red contour boxes with white line segments to illustrate their random orientation patterns. The ordered keratin bundles identified in the fluorescence image as shown in the black box in Fig. 4(a) correspond to coherent and well defined SAXS orientations within the cell interior in the matching region of the dark-field image [white box in Fig. 4(b)]. In contrast, regions outside the cell boundaries display random orientations. We further apply an X-ray intensity threshold to select pixels (white contour) corresponding to the cell regions with densely organized keratin structure, as observed in fluorescence image [black box in Fig. 4(a)]. Only pixels with higher intensity values are included, since their SAXS-derived orientation values are considered more reliable due to improved signal-to-noise ratio. For quantitative comparison, the orientation histograms obtained from fluorescence microscopy and scanning SAXS are calculated within the white contour shown in panel (e), see Fig. 4(f). The orientation values are determined on a pixel-wise basis from *SOAX*-processed fluorescence images and SAXS-derived orientation maps. To ensure spatial correspondence between the two modalities, the fluorescence image is first resampled onto the pixel grid of the X-ray dark-field image, followed by spatial registration, and the corresponding orientation values are considered for the histograms. The resulting orientation distributions from both imaging modalities show good agreement, with the peak of the distribution located around -30° . The strong agreement between the

orientation distributions confirms that keratin filaments constitute the dominant ordered structures in SW-13 cells and demonstrates the utility of the multi-modal correlative imaging approach.

2.4.2. Actomyosin structures in cardiomyocytes

Ordered cytoskeletal assemblies are abundant across many cell types and provide distinct structural signatures that can be probed by complementary imaging modalities. While we first validated our multimodal approach on thick keratin bundles in epithelial cells, our approach is not limited to intermediate filaments. To demonstrate its broader applicability, we apply the multimodal imaging to the contractile actomyosin machinery in cardiomyocytes. Here, thin filaments made of actin and thick filaments made of myosin molecular motors slide past each other and thereby lead to contraction of the cells. These acto-myosin structures are anchored at the Z-disks, which are enriched in α -actinin. In our case, α -actinin is visualized via fusion with citrine fluorescent protein (Härtter *et al.*, 2026; Härtter *et al.*, 2025). Consequently, inverted gray scale fluorescence images of the cardiomyocytes clearly show the Z-disks as dark spots with periodic spacing as shown in Fig. 5(a). Note that, due to the immature state of these iPSC cells, the Z-disks do not yet form well defined transverse bands spanning the entire width of the myofibrils, as observed in the adult human cardiomyocytes. Instead, they appear as shorter and discontinuous structures. Fig. 5(b) shows the corresponding X-ray dark-field image of the selected scan region. A clear contrast between the cytoplasm (green) and nuclei (red) is well visible. Within the cytoplasm, regions exhibiting higher fluorescence intensity, *i.e.* regions with abundant actomyosin and Z-disks, also show enhanced X-ray scattering intensity. Fig. 5(c) shows the orientation map derived from the fluorescence image shown in panel (a) by calculating the structure tensor and deriving the local orientation in each pixel by equation (3) in Section 4.5.4. To validate the utility of the structure tensor for deriving an orientation map, we re-evaluate the orientation of the keratin bundles in the fluorescence images of the SW-13 cells [Fig. 4(a), and Fig. S7 of the supporting information]. The orientation values derived from both *SOAX* and the structure tensor match well.

The corresponding scanning SAXS-derived orientation map (Yu *et al.*, 2026) of the same region is presented in Fig. 5(d). The white outlines indicate the cell contours as obtained from the fluorescence image. We observe dominant orientation features in the regions of clearly resolved Z-disks as compared with the background region outside the white outline and cytoplasmic regions with low scattering intensity [see panel (b)]. Notably, the SAXS-derived orientation reflects the alignment of anisotropic electron density modulations dominated by the highly ordered actomyosin structures. Identical color-coding is used for both modalities to facilitate direct comparison and, interestingly, the orientation maps from both the modalities show similar color distribution. To ensure meaningful quantitative comparison between the two modalities, we therefore select a region where the Z-disks

are clearly resolved and exhibit strong fluorescence intensity, while the SAXS-derived orientation map shows a consistent color in that region [green color, indicated by the box in panels (a)–(d)]. In contrast to the keratin-rich SW-13 cells (Fig. 4), where dense filament bundles extend across large regions of the cytoplasm and give rise to strong X-ray scattering signals throughout the cell, the actomyosin organization in cardiomyocytes is spatially heterogeneous. As a result, the X-ray scattering intensity varies considerably across the cell, and regions with low scattering signal yield less reliable orientation estimates. We therefore restrict the quantitative comparison to regions exhibiting higher X-ray scattering intensity.

Fig. 5(e) shows an overlay of the X-ray dark-field image of the region in the box [panels (a)–(d)], with black line segments for every scan pixel representing the SAXS-determined orientation. The pixels within the white contours are selected based on their higher scattering intensity and correspond to regions containing Z-disks in the fluorescence image [panel (a)]. These higher intensity pixels are included because they provide more reliable SAXS-derived orientation values.

For quantitative comparison, orientation histograms from fluorescence microscopy and SAXS are calculated within the white contour shown in panel (e), see Fig. 5(f). The orientation values are obtained on a pixel-wise basis from the

structure tensor applied to the fluorescence image and from the SAXS-derived orientation map after resampling of the fluorescence image into the pixel grid of the X-ray dark-field image, followed by spatial registration. The resulting orientation distributions from both the modalities show good agreement, with the peak of the distribution located around 0° . This is in line with what we expect for the immature Z-disks in hiPSCs as the structure tensor detects series of α -actinin clusters along the actomyosin structures. In mature cardiomyocytes one would expect more pronounced Z-disks elongate in perpendicular direction to the actomyosin, and, thus, an angular offset of 90° between the distributions derived from the fluorescence images and the scanning SAXS data.

These results demonstrate that our multimodal imaging approach allows for a quantitative assessment of the degree of order in intracellular structures. Fluorescence microscopy provides molecular specificity, whereas scanning SAXS reveals the dominant structural alignment based on electron density contrast, thus highlighting the pronounced complementary of the two methods. The strong correlation between the independently derived orientation maps confirms that both methods consistently identify the same principal axis of filament organization, thereby validating the reliability of orientation analysis across imaging modalities.

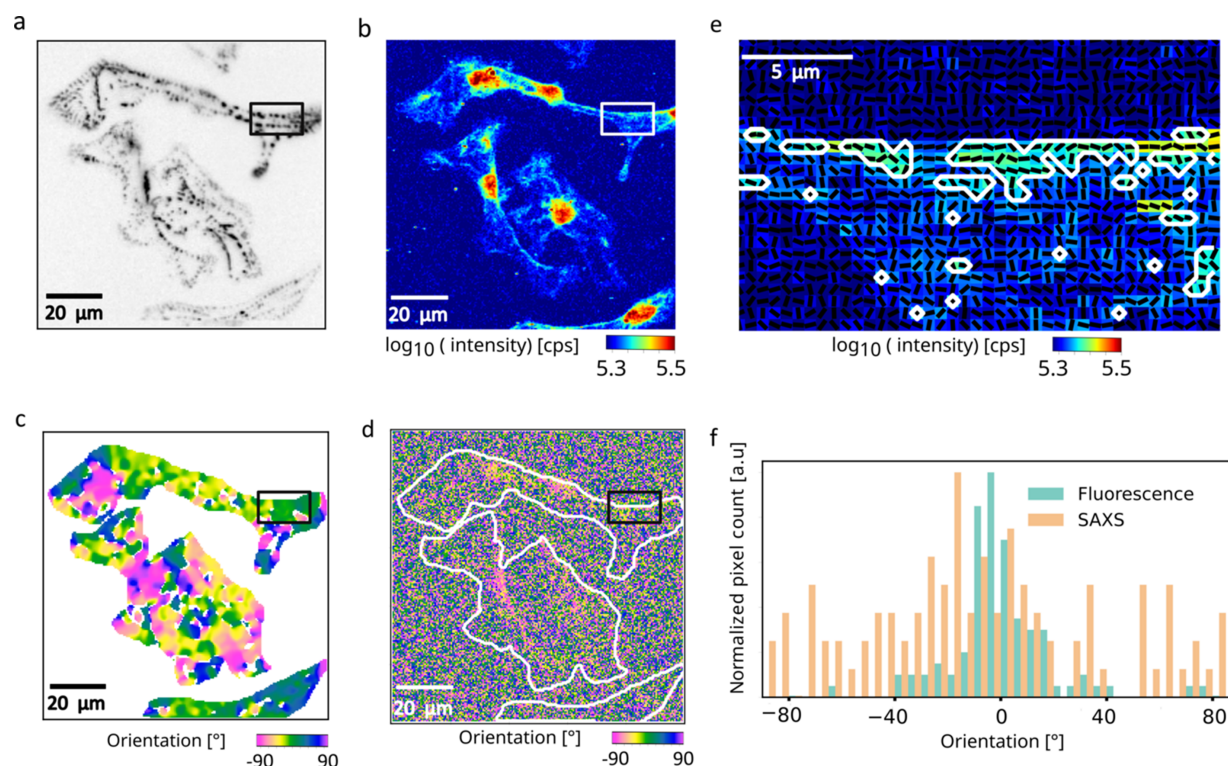


Figure 5

Correlative fluorescence and fast scanning SAXS analysis of sarcomeric organization in hiPSC-ACTN2-citrine-derived cardiomyocytes. (a) Inverted fluorescence image of α -actinin-labeled Z-disks with the periodic sarcomeric arrangement within the cardiomyocytes. The region within the black box highlights a representative area containing Z-disks. (b) The corresponding X-ray dark-field image reveals contrast differences between nucleus and the cytoplasm. (c) Orientation map derived from the fluorescence image of α -actinin-labeled Z-disks (d) SAXS-derived orientation map. The white outlines indicate the cells as shown in panel (a). (e) Overlay of the X-ray dark-field image with black lines representing the SAXS-determined orientation of the region within the black box in panel (a). Pixels within the white contour correspond to areas of high X-ray scattering intensity, where prominent Z-disks are observed in the fluorescence image. (f) Orientation histograms obtained from SAXS and fluorescence analysis for the same region within the white outline as shown in panel (e).

3. Discussion and conclusion

Scanning SAXS is a powerful approach for probing structural organization in biological cells, revealing filament periodicities and bundle architecture with sub-micrometer precision (Fratzl *et al.*, 1997; Lichtenegger *et al.*, 1999; Rinnerthaler *et al.*, 1999; Gourrier *et al.*, 2010; Weinhausen *et al.*, 2012). However, SAXS alone lacks molecular specificity and structural signals cannot be assigned to specific cellular components without complementary imaging. Correlative approaches have partially addressed this gap. Prior work combining cryo-fluorescence based techniques with cryo-soft X-ray tomography, whether using fiducial markers and separate imaging sessions or integrated on-line platforms (Smith *et al.*, 2014; Zhang *et al.*, 2024), has enabled the correlation between molecular identity and internal morphology of biological cells. Similarly, cyro-correlative fluorescence microscopy in combination with synchrotron X-ray fluorescence nanoimaging has provided access to elemental composition in frozen-hydrated cells (Karpov *et al.*, 2026; Ortega *et al.*, 2024). Correlative approaches specifically involving SAXS remain comparatively limited. The foundational work of Bernhardt *et al.* integrated STED fluorescence microscopy with scanning SAXS and X-ray holography, demonstrating the power of combining structural and molecular information, yet exclusively on freeze-dried samples, leaving the extension to hydrated and living biological systems, close to physiological condition, an open challenge (Bernhardt *et al.*, 2018; Bernhardt *et al.*, 2019; Bernhardt *et al.*, 2017). The present work therefore extends this correlative concept to hydrated cellular samples and focuses on the practical integration of fluorescence microscopy with scanning SAXS at a synchrotron beamline. The two cellular systems used here provide complementary levels of structural order: pronounced keratin bundles in SW-13 cells and the more heterogeneous sarcomeric organization of hiPSC-derived cardiomyocytes. This allows us to evaluate the correlative approach across distinct cellular architecture and scattering regimes, from strongly ordered keratin bundles to comparatively weak and heterogeneous sarcomere organization.

Prior SAXS studies on SW-13 cells progressively refined the understanding of keratin organization, from initial freeze-dried sample preparations establishing keratin bundle diameters on the order of tens of nanometers (Weinhausen *et al.*, 2012), through fixed-hydrated and living cell measurements in flow chambers with a 200 μm water layer, to combined ptychography and scanning SAXS yielding quantitative bundle nanostructure (Hémonnot *et al.*, 2016a). However, all were limited by low photon flux, comparatively long acquisition time, and insufficient SAXS signal from hydrated samples and could not reliably extract orientation information. More recently, measurements on fixed-hydrated cells using a thin water layer (20 μm) demonstrated fast scanning with milliseconds exposure times and enabled SAXS-derived orientation mapping through innovative analysis strategies (Yu *et al.*, 2026). However, molecular

specificity through integrated fluorescence imaging remained missing.

For cardiomyocytes, correlative SAXS progressed from cryogenic to close-to-physiological conditions. On freeze-dried neonatal rat cardiomyocytes, home-laboratory fluorescence microscopy combined with scanning SAXS demonstrated orientation mapping (Bernhardt *et al.*, 2017), later extended to integrated STED microscopy and X-ray holography (Bernhardt *et al.*, 2018). Moving to hydrated conditions, scanning SAXS on living iPS-cardiomyocytes in static wet chambers produced X-ray dark-field images, while fixed-hydrated adult mouse cardiomyocytes yielded a detectable sarcomeric diffraction signature (Nicolas *et al.*, 2019). The two strongest equatorial reflections of actomyosin in adult mouse cardiomyocytes were later resolved, with orientation mapping achieved for fixed-hydrated sample preparations (Reichardt *et al.*, 2020). Later work probed sarcomeric diffraction in 3D iPS-cardiomyocytes tissue constructs under hydrated conditions (Javor *et al.*, 2021), and meridional reflections, beyond equatorial peaks, were detected in fixed-hydrated adult mouse cardiomyocytes (Bruns *et al.*, 2024). Despite this progress, two gaps remained unaddressed: integrated fluorescence microscopy on the same sample in hydrated state was not achieved so far and the orientation mapping in weakly ordered systems such as immature iPS-cardiomyocytes remained challenging.

The present work closes both gaps. Our correlative platform integrates high-quality fluorescence microscopy with scanning SAXS in a configuration compatible with hydrated and living cells. The compact microscope hardware adapts to diverse beamline geometries without compromising image quality. The dedicated microfluidic chamber maintains an optimized water layer, thin enough for X-ray transmission, yet sufficiently thick to sustain cells and provide optical access in a physiologically relevant hydrated state. The same chamber enables chemical manipulation of samples during measurements, extending the scope of experiments that can be performed in a single session. Alongside these hardware developments, a dedicated data analysis framework enables quantitative correlation of the multimodal datasets. Together, these aspects provide the practical workflow needed to correlate the fluorescence resolved molecular organization with the spatially resolved SAXS intensity providing X-ray dark-field image and local orientation.

The results validate the platform on two biologically relevant systems. In SW-13 cells, the correlative measurements confirm that keratin filaments constitute the dominant ordered structures contributing to the SAXS signal. The orientation distributions derived independently from fluorescence and from SAXS are in good agreement, establishing a clear link between the molecular identity and nanoscale structural organization. For fixed-hydrated iPS-cardiomyocytes, the results establish a direct correlation between fluorescence-resolved sarcomeric organization and SAXS-derived structural orientation. This is particularly notable given the structural immaturity characteristic of iPS-derived cardiomyocytes. Immature cardiomyocytes cultured on unpatterned

substrates typically show comparatively isotropic structures, making orientation mapping challenging. Thus, this observed correlation supports the sensitivity of the platform and the robustness of the analysis strategy.

The platform opens several directions that were previously inaccessible. Time-resolved measurements on living cells under pharmacological or mechanical stimulation become feasible within the microfluidic chamber. Sequential fluorescence imaging with multiple filter sets can resolve several distinctly labeled components within the same cell, enabling richer multimodal datasets. Integration with additional X-ray modalities like holography, fluorescence or ptychography, is straightforward given the compact and adaptable instrument design. In a broader sense, the approach is not limited to the cytoskeletal systems demonstrated here, but it is applicable to any biological question where nanoscale structural organization and molecular identity must be addressed together under physiological conditions.

4. Materials and methods

4.1. Cell culture and sample preparation

Human adrenal cortex carcinoma-derived SW-13 cells (ATCC CCL-105) (Leibovitz *et al.*, 1973) that stably express fluorescent keratin fusion proteins (HK8-CFP, HK18-YFP) (Strnad *et al.*, 2002; Windoffer *et al.*, 2004) are cultured in high-glucose Dulbecco's Modified Eagle's Medium (DMEM; 4.5 g L⁻¹; D6429, Merck, Darmstadt, Germany) with 10% (v/v) fetal bovine serum (FBS; 10270-106, Gibco, Thermo Fisher Scientific, Waltham, MA, USA) and 1% (v/v) penicillin-streptomycin (pen-strep, 100 U ml⁻¹ penicillin and 0.1 g L⁻¹ streptomycin; 15140122, Gibco), which we refer to as 'SW-13 cell medium'. The cells are maintained at 37°C in a water-saturated atmosphere at 5% CO₂. After one week in culture, geneticin (L0015, Biowest, Nuaille, France) is added (50 mg ml⁻¹) to the culture flask to suppress the cells which are not stably transfected. We refer to these cells as 'SW-13 cells'.

In addition, a sarcomeric Z-band reporter hiPSC-line is used. This cell line has been developed by fusion of the C-terminus of α -actinin 2 gene with fluorescent protein citrine using CRISPR/Cas9 (Härtter *et al.*, 2026). The differentiation of this hiPSC-line to the hiPSC-ACTN2 citrine derived cardiomyocytes is carried out following the protocol by Tiburcy *et al.* (2017). Cell culture flasks are coated with 0.8% (v/v) Matrigel (CLS354230-1EA, Merck) and are incubated at 37°C for 1 h prior to cell culture. The cells are cultured in Roswell Park Memorial Institute (RPMI) 1640 medium with GlutaMAX (61870010, Gibco), with 1% (v/v) pen-strep, 1 mmol L⁻¹ sodium pyruvate and 2% B27 supplement (17504-044, Gibco), which we refer to as 'CM medium'. Cultures are maintained at 37°C, 5% CO₂, and the medium is changed every second day for at least two weeks before sample preparation. We refer to these cells as 'cardiomyocytes'.

For sample preparation, silicon nitride (Si₃N₄) windows (frame size 5 mm × 5 mm, membrane size 1.5 mm × 1.5 mm,

thickness 1 µm, Silson Ltd, Warwickshire, UK) are plasma-treated (50 W, 30 s; Zepto, Diener electronic GmbH & Co. KG, Ebhausen, Germany) and UV-sterilized. For plating the SW-13 cells, the windows are coated with 5% (v/v) fibronectin (F1141, Merck) and incubated at 37°C for 1 h. The cells cultured in the flasks are briefly washed with Dulbecco's phosphate buffered saline (DPBS; D8537, Merck) and detached using 0.25% (v/v) Trypsin – 0.02% (w/v) EDTA (P10-020500, PAN-Biotech GmbH, Aidenbach, Germany). The cells are seeded at a density of 1.5×10^5 cells ml⁻¹ on the treated Si₃N₄ windows. After 24 h, the cells grown on the Si₃N₄ windows are fixed with 4% methanol-free formaldehyde (28906, Thermo Fisher Scientific). For plating the cardiomyocytes, the Si₃N₄ windows are coated with 0.8% (v/v) Matrigel and incubated at 37°C for 1 h. The cells cultured in the flask are briefly washed with DPBS, detached using Accutase (A11105-01, Gibco) with 0.025% Trypsin (15090-046, Gibco) and 20 µg ml⁻¹ DNase I (260913, Calbiochem-Merck), and seeded at 1.5×10^5 cells ml⁻¹ on the treated Si₃N₄ windows. The cultures are maintained for 18 h in CM medium supplemented with 5×10^{-6} mol L⁻¹ Y-27632-2HCl (orb154626, Biorbyt, Durham, NC, USA), followed by only CM medium up to five days before fixation with 4% methanol-free formaldehyde. The fixed cells on Si₃N₄ windows are maintained in DPBS to ensure continuous hydration.

4.2. Fabrication of the microfluidic chamber

A silicon master for the chamber fabrication with a flow channel and supporting structures, made from SU-8, is fabricated by standard photolithography methods, see Fig. S3(a) in the supporting information. Briefly, a 2-inch silicon wafer (MicroChemicals, Ulm, Germany) is spin coated with SU-8 negative photoresist (SU-8 3025; MicroChem, Newton, MA, USA) to achieve a height of 25 µm. The resist is then exposed to UV light through a photomask (Selba, Versoix, Switzerland) containing the dedicated structure design and is developed, see Fig. S2(a) in the supporting information. The silicon master is coated with fluorosilane (1H, 1H, 2H, 2H-perfluorooctyltriethoxysilane, 97%; AB104055, abcr GmbH, Karlsruhe, Germany) by overnight vapor deposition. A PDMS replica for device fabrication is made by mixing polydimethylsiloxane (PDMS; Sylgard 184, Biesterfeld Spezialchemie GmbH, Hamburg, Germany) base and curing agent at a standard ratio of 10:1 (w/w) and curing the mixture on the silicon master, see Fig. S2(b) in the supporting information. Consequently, a single channel with a width of 1500 µm and a height of 25 µm surrounded by repeating cylinder structures of 700 µm diameter with the center-center distance of 1000 µm is obtained in the PDMS replica. These cylindrical structures locally alter the capillary pressure, guiding fluid flow and enabling precise control of capillary filling of the structure by Norland Optical Adhesive 81 (NOA 81, Norland Products, Jamesburg, NJ, USA) in the next step. To do so, the PDMS replica is placed on a Kapton foil (8 µm thickness, SPEX SamplePrep, Metuchen, NJ, USA) on a flat aluminium block to serve as the bottom substrate. 80 µL NOA 81 are pipetted

around the PDMS replica [Fig. S2(c) of the supporting information]. After the capillary filling, the NOA 81 is cured under a UV lamp (365 nm, 2×8 W tubes; Herolab GmbH Herolab GmbH Laborgeräte, Wiesloch, Germany) for two minutes, and the PDMS replica is removed, see Fig. S2(d) in the supporting information. A second Kapton foil is mounted onto a microscope slide ($75 \text{ mm} \times 50 \text{ mm}$, thickness 0.96 to 1.06 mm; Corning Incorporated, NY, USA) using adhesive tape at the edges to ensure flatness. The Kapton surface is plasma activated for 30 s at 50 W (Zepto plasma cleaner, Diener electronic GmbH & Co. KG, Ebhausen, Germany), and adhered to the NOA 81–Kapton assembly, to form the Kapton–NOA 81–Kapton device [Fig. S2(e) in the supporting information]. The device is baked at 65°C for 16 h under light weight ($\sim 30 \text{ g}$) to enhance the bonding strength and ensure proper contact, see Fig. S2(f) in the supporting information.

To provide mechanical support of the device, we bond it to a ring-shaped piece of PDMS (inner diameter 3 cm, outer diameter 5.08 cm, thickness $\sim 1 \text{ cm}$). The PDMS support is attached to the device using a thiol-epoxy-click reaction (Hoang *et al.*, 2016). To do so, the PDMS is plasma activated for 30 s at 50 W, and immediately placed under a desiccator with 40 μL of mercaptosilane (3-mercaptopropyl trimethoxysilane; 175617, Sigma-Aldrich, Merck) on a glass cover slip for vapor deposition for at least 2 h. Following the coating, a two-component epoxy glue (UHU 45705, Bühl, Germany) is applied to the device, and the coated PDMS support is adhered, see Fig. S2(g) in the supporting information. In order to generate a stronger bond, the assembled chamber is compressed under low weight ($\sim 30 \text{ g}$) for at least 3 h. Following the fabrication, a 3.5 mm hole is punched at the center of the flow channel using a biopsy puncher (Plano, Wetzlar, Germany), and one empty Si_3N_4 window is attached using thin double-sided adhesive tape (5 μm ; Nitto, Osaka, Japan). Holes are also punched at the inlet and outlet positions of the microfluidic device with a 0.75 mm-diameter puncher, followed by insertion of tubing (inner diameter: 0.38 mm; outer diameter: 1.09 mm; polyethylene; Becton, Dickinson and Company, NJ, USA) pre-filled with Dulbecco's phosphate buffered saline (DPBS, Merck), see Fig. S2(h) in the supporting information. The tubing is preconnected to a gas tight syringe (2.5 ml, PTFE Luer Lock; Hamilton, Reno, NV, USA) to avoid enclosing of air bubbles. The channel is checked for free fluid flow without clogging using a microfluidic pump (Landgraf Laborsysteme HLL GmbH, Langenhagen, Germany) at a flow rate of $100 \mu\text{L h}^{-1}$. A Si_3N_4 window with cells grown on it is rinsed with ultrapure water to remove remaining salt or dust particles. The frame area around the window is gently blotted, followed by careful alignment of the frame and membrane area with the respective areas of the empty Si_3N_4 window, using double-sided adhesive tape. Additionally, a small amount of cyanoacrylate adhesive (UHU 478747-62, Bühl, Germany) is added around the frame edges of both Si_3N_4 windows for sealing, resulting in the fully assembled microfluidic chamber, see Fig. S2(i) in the supporting information. During the bonding process, a

constant flow rate of $20 \mu\text{L h}^{-1}$ is maintained to prevent dehydration of the cells. Once the glue is cured completely, the flow rate is increased to $100 \mu\text{L h}^{-1}$ and the parts of the chamber holder are assembled, see Fig. S3(c) in the supporting information.

4.3. Immunostaining within the microfluidic chamber

Following the microfluidic chamber assembly, immunostaining is initiated by supplying staining solutions via the inlet. Since the chamber is designed with a single inlet and outlet, the staining solutions are supplied sequentially by exchanging the gastight syringe connected to the inlet. To minimize the introduction of air bubbles during syringe exchange, only the syringe is replaced while keeping the needle–tubing assembly in place. We begin with perfusion of 1% Triton X-100 (3051.2, Carl Roth, Karlsruhe, Germany) in DPBS at a flow rate of $50 \mu\text{L h}^{-1}$ to permeabilize the cell membranes. The Triton solution reaches the cell window after $\sim 25 \text{ min}$. The inlet is then immediately switched to DPBS and rinsed at $100 \mu\text{L h}^{-1}$ for 30 min. The DPBS reaches the cell window after $\sim 12.5 \text{ min}$, providing a net Triton incubation time of $\sim 2.5 \text{ min}$. To block non-specific binding, 3% bovine serum albumin (130-091-376, MACS BSA Stock Solution; Miltenyi Biotec) in DPBS is perfused at $100 \mu\text{L h}^{-1}$ for 30 minutes. The flow is then stopped, and the sample is incubated under static conditions for 45 min. Following incubation with the BSA solution, a primary antibody against K8 keratin, Troma I ($0.5 \mu\text{g ml}^{-1}$ in 3% BSA; AB531826, DSHB, Iowa, USA), is introduced into the chamber at a flow rate of $50 \mu\text{L h}^{-1}$ for 40 min. The flow is then stopped, and the sample is incubated for 1.5 h under static conditions. The chamber is subsequently rinsed with DPBS at $100 \mu\text{L h}^{-1}$ for 30 min. Secondary antibody staining is performed using $1 \mu\text{g ml}^{-1}$ goat anti-rat IgG conjugated with Alexa Fluor Plus 647 (A32733TR, Thermo Fisher Scientific), which is perfused at $50 \mu\text{L h}^{-1}$ for 30 min. The flow is again stopped, and the sample is incubated for an additional hour. Finally, the sample is rinsed with DBPS at a flow rate of $100 \mu\text{L h}^{-1}$ for 30 min. All imaging before and after immunostaining is performed on an inverted microscope (IX83, Olympus/Evident) using an IX3-FGFPXL filter set (excitation: 460–480 nm; emission: 495–540 nm; dichroic: 490 nm; Olympus/Evident), an ET-Cy5 filter set (excitation: 590–650 nm; emission: 662–738 nm; dichroic: 660 nm; Olympus/Evident) and a $20\times$ objective (NA 0.7, UCPLFLN20X, Olympus/Evident).

4.4. Integrated fast scanning SAXS and fluorescence microscopy

The combined fast scanning SAXS and visible light fluorescence microscopy experiments are performed at the nano-branch (experimental hut 3, EH3) of the ID13 beamline at the European Synchrotron (ESRF, Grenoble, France). The workflow comprises the microfluidic chamber assembly, identification of regions of interest (ROIs) based on fluorescence microscopy, and targeted scanning SAXS measurements of the same regions. Fluorescence microscopy is used to select

cells exhibiting ordered cytoskeletal structures. The keratin structures in SW-13 cells and the Z-disks in cardiomyocytes are imaged with a YFP filter-set and 100 ms exposure time, see Table S1 in the supporting information. The motor positions of each ROI are stored to ensure repositioning during scanning SAXS measurements.

Prior to scanning SAXS measurements, the microscope is retracted along the y -axis by ~ 125 cm to clear the beam path. The undulator beam is monochromatized by a channel-cut Si (111) monochromator to a photon energy of 15.0 keV. The beam is prefocused by beryllium parabolic refractive lenses (Be-CRLs). The X-rays are focused by a multilayer Laue lens (MLL; focal length 90 mm, from ESRF long-term proposal collaboration MI-1463) and spatially cleaned by a square-shaped $40\text{ }\mu\text{m} \times 40\text{ }\mu\text{m}$ PtIr aperture (order-sorting aperture, OSA), resulting a beam spot size of about $250\text{ nm} \times 250\text{ nm}$ (horizontal $\times$ vertical) at the sample position inside the microfluidic chamber. A helium-filled flight tube is placed between the microfluidic chamber and the SAXS beamstop (made of lead, $\sim 250\text{ }\mu\text{m}$ diameter), leaving only a few millimeters of air path on both sides of the flight tube. This configuration helps minimizing the background contribution from air scattering. The X-ray scattering patterns are recorded using an Eiger X 4M detector (Dectris, Baden, Switzerland) with 2070×2167 pixels (4 megapixels) and a pixel size of $75\text{ }\mu\text{m} \times 75\text{ }\mu\text{m}$ located at a sample-to-detector distance of about 1.9 m. This distance is calibrated using AgBeh powder placed in a empty chamber. In fast scanning SAXS mode, data are acquired using continuous line scans with constant stage motion. A $100\text{ }\mu\text{m} \times 100\text{ }\mu\text{m}$ scan area with $0.5\text{ }\mu\text{m} \times 0.5\text{ }\mu\text{m}$ step size (40000 scattering patterns) requires ~ 4 min at 5 ms exposure per position (used for the cardiomyocytes) or ~ 3 min at 2 ms exposure per position (used for the SW-13 cells). At the end of each scan line, the recording is paused while the piezo stage moves to the start of the next line. During this time, the sample is still being exposed.

4.5. Data analysis

4.5.1. SAXS data

We follow the analysis method described by Yu *et al.* (2026). The X-ray dark-field image is obtained by integrating the total scattering intensity within an optimized q -range of 0.19–0.39 nm^{-1} for each scattering pattern. This approach effectively denoises the data and enhances contrast in the dark-field image. From the collected scattering patterns, the local orientation is computed by evaluating the circular mean of the azimuthal intensity distribution after background subtraction and masking. This analysis yields spatially resolved maps of the predominant scattering orientation, representing the local structural organization within the sample.

4.5.2. Spatial image registration

The fluorescence images are rescaled by interpolation to match the pixel size of the X-ray dark-field images to ensure a common spatial sampling grid. Image registration is performed on the rescaled fluorescence image and the X-ray

dark-field image based on cross-correlation in Fourier space (Guizar-Sicairos *et al.*, 2008). The displacement (Δy , Δx) is computed with subpixel precision using an upsampling factor of 20. The detected translation is applied using the Fourier shift theorem. In this framework, a spatial shift in real space corresponds to a phase modulation in Fourier space, $I'(\mathbf{q}) = I(\mathbf{q})\exp(-i\mathbf{q} \cdot \Delta\mathbf{r})$ where $I(\mathbf{q})$ denotes the Fourier transform of the image and $\Delta\mathbf{r}$ denotes the displacement. The registered image is obtained by inverse Fourier transformation.

4.5.3. Filament-tracing based orientation mapping

The orientation of the keratin bundles in SW-13 cells as observed in the fluorescence images is quantified by filament detection followed by azimuthal angle analysis. The filaments are traced using the software package *SOAX*. We set the parameter 'init-z' to false, set the ridge-threshold to 0.008 and the minimum filament length to 10 pixels. All other parameters are set to the default values. The traced filament coordinates are exported and further analyzed using a custom Python script to determine local filament orientations.

For each filament detected by *SOAX*, the azimuthal angle is calculated from the local tangent direction between consecutive filament points. Specifically, for each segment defined by two neighboring coordinates (x_1, y_1) and (x_2, y_2) the in-plane angle is computed as

$$\theta = \tan^{-1}\left(\frac{-\Delta y}{\Delta x}\right), \quad (1)$$

where $\Delta x = x_2 - x_1$, and $\Delta y = y_2 - y_1$. This angle is then mapped onto the symmetric range $[-90^\circ, +90^\circ]$ to represent orientations (*i.e.* directions without polarity). Hence, the azimuthal angle corresponds to the local in-plane orientation of each filament segment with respect to the horizontal axis (x -axis) of the image, *i.e.* the horizontal x -axis is considered as 0° , and the vertical y -axis is considered to be $+90^\circ$.

4.5.4. Structure-tensor based orientation mapping

We apply a structure tensor based approach (Rezakhaniha *et al.*, 2012) to evaluate the orientation of keratin filaments in SW-13 cells and labeled Z-disks in cardiomyocytes. Unlike filament tracing methods, which require continuous filamentous structures, the structure tensor estimates local orientations directly from image intensity gradients and therefore does not rely on explicit segmentation. This makes it particularly suitable for structures like Z-disks. For an image intensity distribution $I(x, y)$, the spatial intensity gradients $I_x = \partial I / \partial x$ and $I_y = \partial I / \partial y$ are computed after Gaussian presmoothing with a gradient scale σ . The local structure tensor is defined as

$$\mathbf{J} = \begin{pmatrix} A_{xx} & A_{xy} \\ A_{xy} & A_{yy} \end{pmatrix} = G_\rho \begin{pmatrix} I_x^2 & I_x I_y \\ I_x I_y & I_y^2 \end{pmatrix}, \quad (2)$$

where G_ρ denotes Gaussian averaging with the integration scale ρ . The structure tensor components (A_{xx} , A_{xy} , A_{yy}) thus represent locally averaged second-order intensity gradients. The local orientation angle is computed as

$$\theta = \frac{1}{2} \tan^{-1} \left[\frac{2A_{xy}}{A_{yy} - A_{xx}} \right]. \quad (3)$$

Here, θ corresponds to the direction of the dominant eigenvector of the structure tensor and yields the local orientation of image features. For our analysis, the gradient scale is set to $\sigma = 1.0$ pixels and the integration scale is set to $\rho = 2.0$ pixels. The gradient scale determines the spatial extent of structures contributing to the derivative, whereas the integration scale controls spatial averaging of the tensor components, therefore improving robustness against noise. To restrict the analysis to regions exhibiting a dominant orientation, we compute the local anisotropy from the eigenvalues ($\lambda_1 \geq \lambda_2$) of the structure tensor $\mathbf{J}$ as

$$c = \frac{\lambda_1 + \lambda_2}{\lambda_1 - \lambda_2}. \quad (4)$$

This value ranges from 0 (isotropic, no preferred direction) to 1 (highly oriented). Pixels with anisotropy < 0.2 are excluded to guarantee reliable orientation estimation. To demonstrate the utility of the approach, we apply this procedure to synthetic data showing stripe patterns with different orientations (horizontal, vertical and diagonal) (Fig. S8 of the supporting information).

4.5.5. Orientation histograms

Pixel-wise orientation values are extracted from the spatially registered fluorescence images and the SAXS-derived orientation maps. For quantitative comparison, both orientation distributions are normalized such that the integral equals 1. The normalized data are rebinned over the angular range $[-90^\circ, +90^\circ]$ into 36 bins (5° width) by weighted integration within each interval, followed by renormalization. The bin centers, defined as the midpoints of the angular intervals, are used to represent the mean orientation in the comparison plots.

5. Author contributions

SK and MB conceived the project, and SK supervised the study. MS designed and built the fluorescence microscope. BY designed and fabricated the microfluidic chamber. MS, BY, RMS, MB, and SK performed the experiments. PL assisted in designing and building the microscope. MT and WHZ provided the hiPSC cell line and associated protocols. UR performed the cell culture and assisted with sample preparation. MS performed the data analysis with assistance from BY. MS and SK wrote the first draft of the manuscript, and all authors contributed to revising and finalizing the manuscript.

Acknowledgements

We are grateful for the long term proposal beam time (SC-5436) granted by the ESRF (The European Synchrotron, Grenoble, France), and the excellent support of the ID 13 staff and the PSCM (Partnership for Soft Condensed Matter),

specifically Pierre Lloria, and Peter van der Linden. We thank Peter Gawlitza and Sven Niese for providing MLL-lens prototypes used for these experiments and Wonhee Lee for helpful suggestions on the microfluidic chamber design. We further thank Claudia Geisler for fruitful discussions regarding the microscopy. Open access funding enabled and organized by Projekt DEAL.

Conflict of interest

The authors declare that there are no conflicts of interest.

Data availability

The datasets generated and processed for the current study have been deposited to the GRO.data repository and are available at <https://doi.org/10.25625/3RFST3>.

Funding information

This work was financially supported by the German Federal Ministry of Research, Technology and Space (BMFTR) under grants No. 05K22MG3 and 05K25MGA (to SK) and the German Research Foundation (DFG): project-ID 449750155-RTG 2756, projects A2, A7 (to SK) and under Germany's Excellence Strategy – EXC 2067, 'Multiscale Bioimaging: from Molecular Machines to Networks of Excitable Cells' (MBExC, grant No. EXC 2067/1-390729940, to SK and WHZ) and in the framework of DAPHNE4NFDI (grant No. 460248799), to SK. WHZ is additionally supported by the DZHK (German Center for Cardiovascular Research), the German Federal Ministry for Science and Education (BMBF FKZ 161L0250A), and the Foundation Leducq (20CVD04). We acknowledge support by the Open Access Publication Funds of the University of Göttingen.

References

- Albers, J., Svetlove, A. & Duke, E. (2024). *J. Cell Sci.* **137**, jcs261953.
- Bernhardt, M., Nicolas, J.-D., Eckermann, M., Eltzner, B., Rehfeldt, F. & Salditt, T. (2017). *New J. Phys.* **19**, 013012.
- Bernhardt, M., Nicolas, J.-D., Osterhoff, M., Mittelstädt, H., Reuss, M., Harke, B., Wittmeier, A., Sprung, M., Köster, S. & Salditt, T. (2018). *Nat. Commun.* **9**, 3641.
- Bernhardt, M., Nicolas, J.-D., Osterhoff, M., Mittelstädt, H., Reuss, M., Harke, B., Wittmeier, A., Sprung, M., Köster, S. & Salditt, T. (2019). *J. Synchrotron Rad.* **26**, 1144–1151.
- Bruns, H., Czajka, T. S., Sztucki, M., Brandenburg, S. & Salditt, T. (2024). *Biophys. J.* **123**, 3024–3037.
- Cassini, C., Wittmeier, A., Brehm, G., Denz, M., Burghammer, M. & Köster, S. (2020). *J. Synchrotron Rad.* **27**, 1059–1068.
- Fantoni, S., Brombal, L., Cardarelli, P. & Baruffaldi, F. (2025). *Proc. R. Soc. A Math. Phys. Eng. Sci.* **481**, 20250500.
- Fratzl, P., Jakob, H. F., Rinnerthaler, S., Roschger, P. & Klaushofer, K. (1997). *J. Appl. Cryst.* **30**, 765–769.
- Fratzl, P., Klaushofer, K., Gupta, H. S., Roschger, P., Zizak, I., Fratzl-Zelman, N. & Nader, A. (2003). *Calcif. Tissue Int.* **72**, 567–576.
- Gaiser, S., Deyhle, H., Bunk, O., White, S. N. & Müller, B. (2012). *Biointerphases* **7**, 4.

- Ghazal, A., Lafleur, J. P., Mortensen, K., Kutter, J. P., Arleth, L. & Jensen, G. V. (2016). *Lab Chip* **16**, 4263–4295.
- Gourrier, A., Li, C., Siegel, S., Paris, O., Roschger, P., Klaushofer, K. & Fratzl, P. (2010). *J. Appl. Cryst.* **43**, 1385–1392.
- Guizar-Sicairos, M., Thurman, S. T. & Fienup, J. R. (2008). *Opt. Lett.* **33**, 156–158.
- Härtter, D., Hauke, L., Driehorst, T., Long, Y., Bao, G., Primeßnig, A., Berečić, B., Cyganek, L., Tiburcy, M., Schmidt, C. F. & Zimmermann, W.-H. (2025). Preprint, <https://www.biorxiv.org/content/early/2025/05/04/2025.04.29.650605>.
- Härtter, D., Hauke, L., Driehorst, T., Nishi, K., Zimmermann, W.-H. & Schmidt, C. F. (2026). *eLife* **13**, RP97321.
- Hémonnot, C. Y. J. & Köster, S. (2017). *ACS Nano* **11**, 8542–8559.
- Hémonnot, C. Y. J., Ranke, C., Saldanha, O., Graceffa, R., Hagemann, J. & Köster, S. (2016b). *ACS Nano* **10**, 10661–10670.
- Hémonnot, C. Y. J., Reinhardt, J., Saldanha, O., Patommel, J., Graceffa, R., Weinhausen, B., Burghammer, M., Schroer, C. G. & Köster, S. (2016a). *ACS Nano* **10**, 3553–3561.
- Hoang, M. V., Chung, H.-J. & Elias, A. L. (2016). *J. Micromech. Microeng.* **26**, 105019.
- Javor, J., Ewoldt, J. K., Cloonan, P. E., Chopra, A., Luu, R. J., Freychet, G., Zhernenkov, M., Ludwig, K., Seidman, J. G., Seidman, C. E., Chen, C. S. & Bishop, D. J. (2021). *Microsyst Nanoeng* **7**, 10.
- Karpov, D., Cuau, L., Shishkov, R., Gramaccioni, C., Dallerba, E., Schwehr, B. J., Ellison, G., Hackett, M. J., Plush, S. E., Massi, M., Lerouge, F., Cloetens, P. & Bohic, S. (2026). *ACS Nano* **20**, 7401–7413.
- Köster, S. & Pfohl, T. (2012). *Mod. Phys. Lett. B* **26**, 1230018.
- Leibovitz, A., McCombs, W. M., Johnston, D., McCoy, C. E. & Stinson, J. C. (1973). *J. Natl Cancer Inst.* **51**, 691–697.
- Lichtenegger, H., Reiterer, A., Stanzl-Tschegg, S. & Fratzl, P. (1999). *J. Struct. Biol.* **128**, 257–269.
- Nicolas, J.-D., Bernhardt, M., Schlick, S. F., Tiburcy, M., Zimmermann, W.-H., Khan, A., Markus, A., Alves, F., Toischer, K. & Salditt, T. (2019). *Prog. Biophys. Mol. Biol.* **144**, 151–165.
- Ortega, R., Fernández-Monreal, M., Pied, N., Roudeau, S., Cloetens, P. & Carmona, A. (2024). *Chem. Biomed. Imaging* **2**, 744–754.
- Piazza, V., Weinhausen, B., Diaz, A., Dammann, C., Maurer, C., Reynolds, M., Burghammer, M. & Köster, S. (2014). *ACS Nano* **8**, 12228–12237.
- Priebe, M., Bernhardt, M., Blum, C., Tarantola, M., Bodenschatz, E. & Salditt, T. (2014). *Biophys. J.* **107**, 2662–2673.
- Reichardt, M., Neuhaus, C., Nicolas, J.-D., Bernhardt, M., Toischer, K. & Salditt, T. (2020). *Biophys. J.* **119**, 1309–1323.
- Rezakhaniha, R., Agianniotis, A., Schrauwen, J. T. C., Griffa, A., Sage, D., Bouten, C. V. C., van de Vosse, F. N., Unser, M. & Stergiopulos, N. (2012). *Biomech. Model. Mechanobiol.* **11**, 461–473.
- Rinnerthaler, S., Roschger, P., Jakob, H. F., Nader, A., Klaushofer, K. & Fratzl, P. (1999). *Calcif. Tissue Int.* **64**, 422–429.
- Smith, E. A., Cinquin, B. P., Do, M., McDermott, G., Le Gros, M. A. & Larabell, C. A. (2014). *Ultramicroscopy* **143**, 33–40.
- Strnad, P., Windoffer, R. & Leube, R. E. (2002). *J. Cell Sci.* **115**, 4133–4148.
- Tesch, W., Eidelman, N., Roschger, P., Goldenberg, F., Klaushofer, K. & Fratzl, P. (2001). *Calcif. Tissue Int.* **69**, 147–157.
- Tiburcy, M., Hudson, J. E., Balfanz, P., Schlick, S., Meyer, T., Chang Liao, M. L., Levent, E., Raad, F., Zeidler, S., Wingender, E., Riegler, J., Wang, M., Gold, J. D., Kehat, I., Wettwer, E., Ravens, U., Dierickx, P., van Laake, L. W., Goumans, M. J., Khadjeh, S., Toischer, K., Hasenfuss, G., Couture, L. A., Unger, A., Linke, W. A., Araki, T., Neel, B., Keller, G., Gepstein, L., Wu, J. C. & Zimmermann, W. H. (2017). *Circulation* **135**, 1832–1847.
- Weinhausen, B. (2013). *Scanning X-ray Nano-Diffraction on Eukaryotic Cells: From Freeze-Dried to Living Cells*. PhD thesis, Georg-August-Universität Göttingen, Germany.
- Weinhausen, B. & Köster, S. (2013). *Lab Chip* **13**, 212–215.
- Weinhausen, B., Nolting, J.-F., Olendrowitz, C., Langfahl-Klabes, J., Reynolds, M., Salditt, T. & Köster, S. (2012). *New J. Phys.* **14**, 085013.
- Weinhausen, B., Saldanha, O., Wilke, R. N., Dammann, C., Priebe, M., Burghammer, M., Sprung, M. & Köster, S. (2014). *Phys. Rev. Lett.* **112**, 088102.
- Windoffer, R., Wöll, S., Strnad, P. & Leube, R. E. (2004). *Mol. Biol. Cell*, **15**, 2436–2448.
- Xia, Y. & Whitesides, G. M. (1998). *Angew. Chem. Int. Ed.* **37**, 550–575.
- Xu, T., Vavylonis, D., Tsai, F.-C., Koenderink, G. H., Nie, W., Yusuf, E., Lee, I. J., Wu, J.-Q. & Huang, X. (2015). *Sci. Rep.* **5**, 9081.
- Yu, B., Sinha, M., Da Silva, R. M., Rölleke, U., Burghammer, M. & Köster, S. (2026). *J. Synchrotron Rad.* **33**, 777–787.
- Zhang, C., Guan, Y., Tao, X., Tian, L., Chen, L., Xiong, Y., Liu, G., Wu, Z. & Tian, Y. (2024). *Opt. Express* **32**, 27508–27518.