A waveguide imaging platform for live-cell TIRF imaging of neurons over large fields of view

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Abstract

Large fields of view (FOVs) in total internal reflection fluorescence microscopy (TIRFM) via waveguides have been shown to be highly beneficial for single molecule localisation microscopy on fixed cells [1,2] and have also been demonstrated for short-term live-imaging of robust cell types [3–5], but not yet for delicate primary neurons nor over extended periods of time. Here, we present a waveguide-based TIRFM set-up for live-cell imaging of demanding samples. Using the developed microscope, referred to as *the ChipScope*, we demonstrate successful culturing and imaging of fibroblasts, primary rat hippocampal neurons and axons of *Xenopus* retinal ganglion cells (RGCs). The high contrast and gentle illumination mode provided by TIRFM coupled with the exceptionally large excitation areas and superior illumination homogeneity offered by photonic waveguides have potential for a wide application span in neuroscience applications.
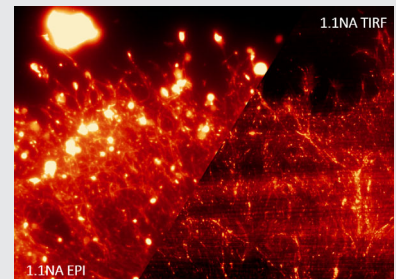
TIRFM provides an effective means for the spatially confined illumination of a sample close to the coverslip/substrate via evanescent fields [6–8]. It provides particular advantages for fluorescence imaging as out of focus signal is intrinsically avoided leading to high signal to noise ratios and image contrast. In addition to the molecular specificity afforded by fluorescence imaging and image contrast, TIRFM reduces the overall illumination dose on the sample. This minimises phototoxicity, making TIRF the method of choice for many live-

cell imaging applications with delicate samples such as live neurons [9].

TIRFM is usually accomplished by using a large numerical aperture (NA) objective lens for both the excitation and the detection paths. Unfortunately, the high magnification of lenses required for TIRFM limits the accomplishable FOVs and imaging throughput, but also which studies, both qualitative and quantitative, are possible to perform. For instance, in conventional TIRF, it would not be possible to measure the cellular response to a drug treatment or other stimulus over a large population of cells or even across a single polarised cell that spans wider than the conventional TIRF FOV. This inhibits the acquisition of statistically significant biological data from studies that rely on correlation in both space and time, as these are not simultaneously accessible. Our chip-TIRF imaging platform allows for the generation of large data sets which capture the correlation in both space and time, which facilitates the obtaining of statistically significant results also for studies where the time dimension is of essence. Moreover, if samples are fast-moving, cellular events become close to impossible to follow using a small FOV. For example, in Reference [10], elongated tubules in hippocampal neurons are reported to move extremely fast: on average 4.5 μm/s.

The restriction on the TIRF FOV is removed if waveguides are used for TIRF illumination and in principle, arbitrarily large areas could be achieved through appropriately designed waveguide geometries (width and length). Because the excitation and the detection paths are completely decoupled from one another, full flexibility in choice of the imaging objective lens is retained, allowing for control over the FOV size, as illustrated on fibroblasts in Figure S1. In the so-called *ChipScope* microscopy system[1], multiple colours can be admitted simultaneously into the photonic chip, enabling the simultaneous TIRF excitation of multiple fluorophores (see Figure S2).

Waveguides have previously been shown to be a viable growth substrate for cell culture [3, 4], but to fully exploit the gentle TIRF illumination for live-cell image applications, especially in the neurosciences, additional considerations and adaptations must be made to maintain the cells alive under suitable conditions. The scope of this work was to adapt a waveguide TIRF microscopy set-up for the imaging of sensitive cell types like primary neurons, and to develop means of performing measurements on primary cell-cultures on photonic chips. These are demanding and challenging cells to grow in general and especially on waveguide materials (illustrated in Figure S3), as the surface properties are different compared to cover glasses, which are currently standard for neuronal culturing.

Cultured neurons from *Xenopus laevis* (African clawed frog) are viable at room temperature under atmospheric levels of oxygen and CO_2, making them an attractive and practical choice for studies requiring prolonged live imaging and a suitable initial test specimen for the *ChipScope*. Retinal neurons cultured from eye primordia are an interesting model system where both TIRFM and large FOV are highly beneficial [11]. The growth cones at the tip of extending axons are flat, hand-shaped structures that are responsive to extracellular chemical and mechanical stimuli and support axon pathfinding during embryonic development [12]. To image the growth of live RGC axons in culture, we explored the capabilities of our waveguide imaging platform in simultaneously capturing tens of growth cones of far-reaching axons from explanted *Xenopus* eye primordia. Different from previous waveguide imaging implementations, we employed water dipping objective lenses, which greatly facilitate high-resolution live-cell imaging and permit access to the sample during imaging, e.g. to optimise labelling conditions or study the response of different treatments in actu [13]. The results of imaging of filamentous actin in live developing axons and growth cones of RGC are shown in Figure 1. The benefit of TIRFM over episcopic (EPI) illumination is apparent when comparing identifiable single cortical filaments of growth cones, as shown in Figure 1, panels D-F. Additionally, the vastly increased FOV simplifies and improves both qualitative and quantitative analyses.

While *Xenopus* neurons can be imaged under ambient conditions, mammalian cells require 37°C and 5% CO_2. To allow for long-term imaging of mammalian neurons under physiologically relevant conditions, we equipped the *ChipScope* with a heater system and a custom-made environmental chamber. As laser coupling together with its delicate piezo stage electronics precludes the use of common commercially available microscope stage incubators on the *Chipscope*, we custom designed a chamber connected to a commercial (Okolab) stage top incubator system, supplying 5% CO_2 and high humidity. Our chamber is made of transparent, flexible and low heat conductivity low-density polyethylene (LDPE) thermoplastic, which can be easily cut and stretched to tightly fit around all necessary microscope components, while maintaining easy access for changing sample or objective through a zip open/close mechanism (Figure 2E, F). A heating strip and temperature sensor are fitted to the sample holder to maintain the chip at 37°C during imaging experiments. Further details are provided in Suppl. Note 1.

Standard protocols for culturing rat hippocampal neurons [14] require tall, slim chambers (as in Figure 2B, second row). These culture chambers are incompatible with upright microscopes featuring short working distance,
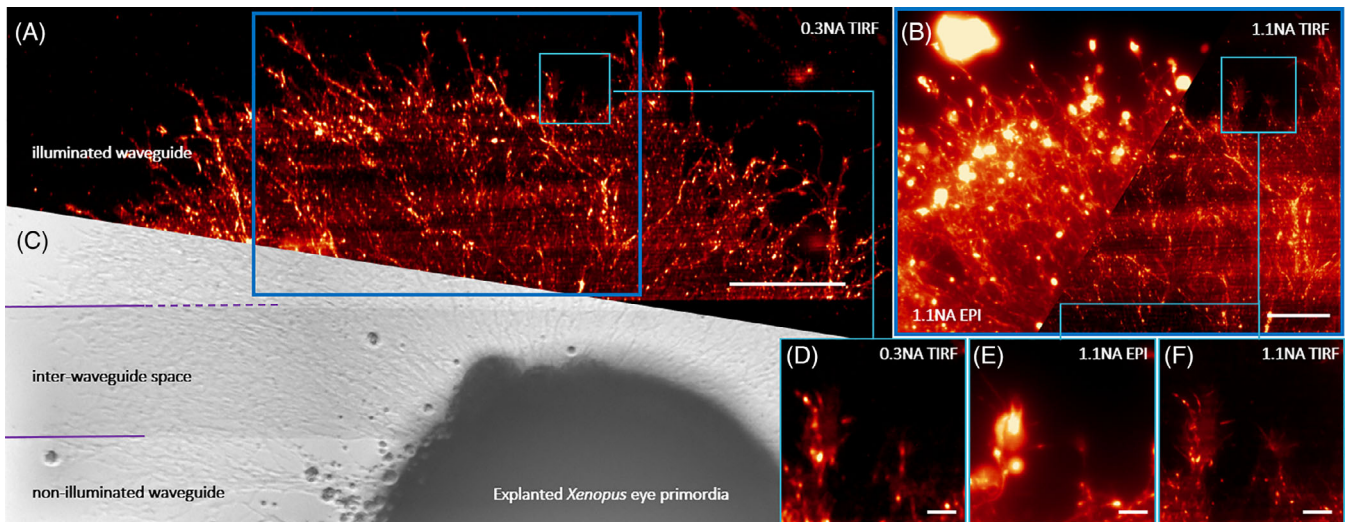
FIGURE 1 Chip microscopy is a method for imaging of large areas of live Xenopus RGC axons in TIRF. All panels show images of the same living *Xenopus* eye explant cultured on chip and labelled with SiR-Actin. A, Overview image captured using 0.3NA water dipping objective in both TIRF (upper panel) and BF (lower panel) mode. B, Overview image captured using 1.1NA water dipping objective using EPI (left panel) and TIRF (right panel) illumination modes. D-F, Excerpts from region indicated in B, comparing available growth cone details for different NAs in TIRF and EPI illumination modes. TIRF illumination together with 1.1NA in panel f reveals the most identifiable single cortical filaments. TIRF images were obtained via summation of 100 frames illuminated by different waveguide illumination modes. Scale bars: A, 100 µm; B, 50 µm and D-F, 10 µm
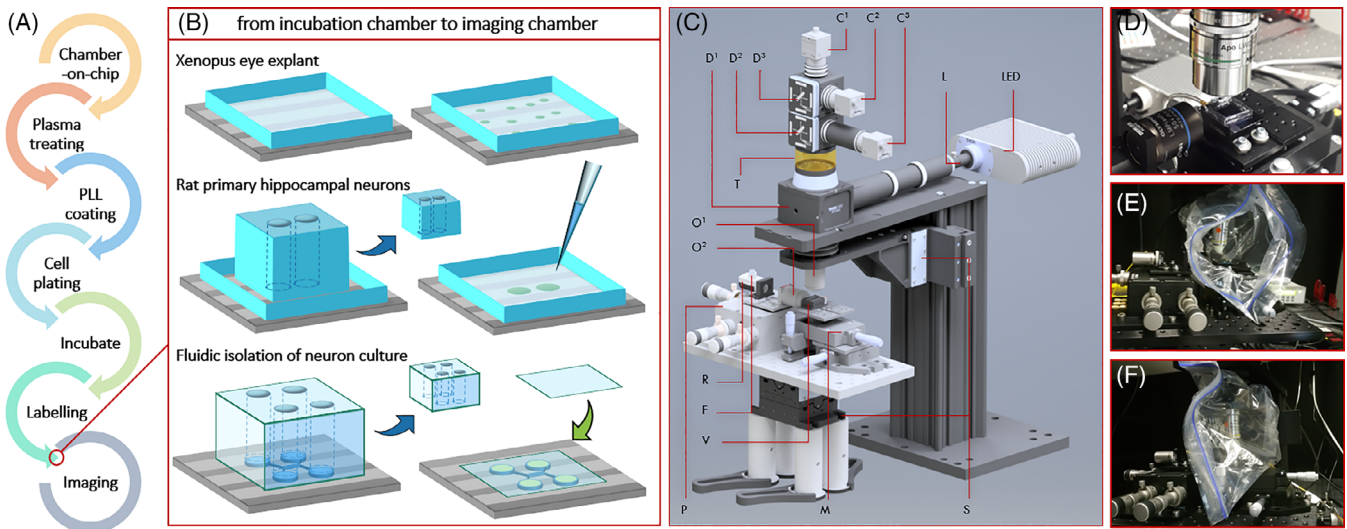

FIGURE 2 Chip microscopy can be adapted for live-cell imaging and various cell culture approaches. A, Experimental flowchart. B, Photonic chip preparation for cell culture and imaging. *Top*: chambers for *Xenopus* retinal ganglion cells. The chambers are about 20 mm by 20 mm wide and 3 mm tall; *Middle*: chambers for rat hippocampal neurons. The outer chamber is as above, while the inner block is about 10 mm by 15 mm wide and 10 mm tall. The inner circular wells containing neurons and cell culture medium are of diameter 6 mm; *Bottom*: two layers of PDMS for cultivating neurons in microgrooves. The dimensions are as above, but with a rectangular PDMS block of about 15 mm by 15 mm sustaining the circular wells. The thin bottom layer containing the microchannels remains for imaging with a coverslip on top to reduce evaporation. C, *ChipScope* model. This upright microscope enables TIRF, EPI, and BF imaging with up to three colours simultaneously. C1-3: cameras, D1-3: dichroic mirrors, T: tube lens, L: liquid lightguide, LED: 4-colour LED combiner, O1-2: objectives, R: reflective collimator, F: fibre, V: vacuum-stage, P: piezo-stage, M: micrometre long-travel stage, S: sample *xyz*-stage. A detailed description of the optical set-up is provided in Suppl. Note 1. D, Microscope stage with waveguide chip and imaging chamber prepared for use with water dipping objective. The horizontal objective is for laser coupling into the waveguides. E, ChipScope with open incubation chamber for easy access to sample and objective. F, Closed incubator supplied with high humidity and 5% CO_2 from a conventional stage top incubator
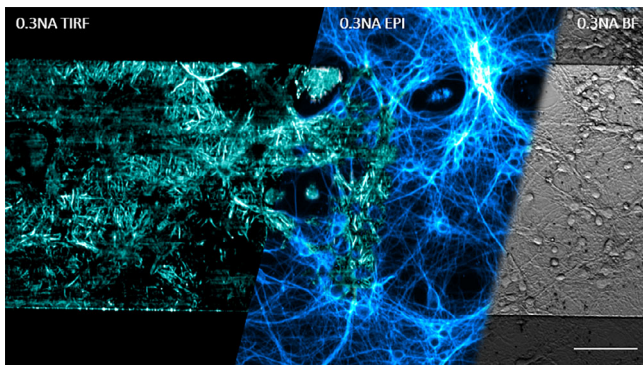
FIGURE 3 Rat hippocampal neurons can be cultured on chip for weeks and imaged live for hours in ChipScope incubator. All panels show live-cell imaging of neurons labelled with SiR-Tubulin on adjacent regions of the same waveguide providing a 400 μm wide TIRF excitation area. The length of the *imaged* TIRF region is limited by the system magnification and camera chip size, in this case giving a total TIRF field of view of 400 μm × 847 μm. *Left*: TIRF image obtained by mode-averaging as in Figure 1. *Middle*: Single plane EPI image overlaid with part of the corresponding TIRF image. *Right*: BF image. Scale bar: 100 μm

high-NA objectives, while inverted microscopy is rendered impractical by the opaque base layer of silicon that forms a supporting platform for the waveguides. We

solved this difficulty via separate custom-made wells for culturing and imaging, as displayed in Figure 2B. After growing the neurons on-chip in their preferred polydimethylsiloxane (PDMS) cell culture wells, we exchanged the tall PDMS blocks right before imaging for wider and lower "fences" adapted for the particular waveguide chip and imaging objective of choice. To monitor the on-chip hippocampal cultures in the time between excision and laser lab TIRF imaging, we built a simple upright microscope that could conveniently be fitted on a standard biological lab bench, see Figure S4. A flowchart of our on-chip sample preparation is shown in Figure 2A,B and the imaging set-up in Figure 2C,F.

The satisfactory performance of this incubator was validated through longer-term live-cell imaging of delicate primary hippocampal neurons (excised from rat embryos). The results, displayed in Figure 3, show the microtubule network imaged in TIRF, EPI and brightfield (BF) mode (from left to right). After 1.5 hours of imaging, the primary neurons were observed to be in a healthy condition (Figure S5). To the best of our knowledge, this is the first chip-based imaging system with incubation chamber that has been successfully adapted for live-cell imaging of mammalian neurons.
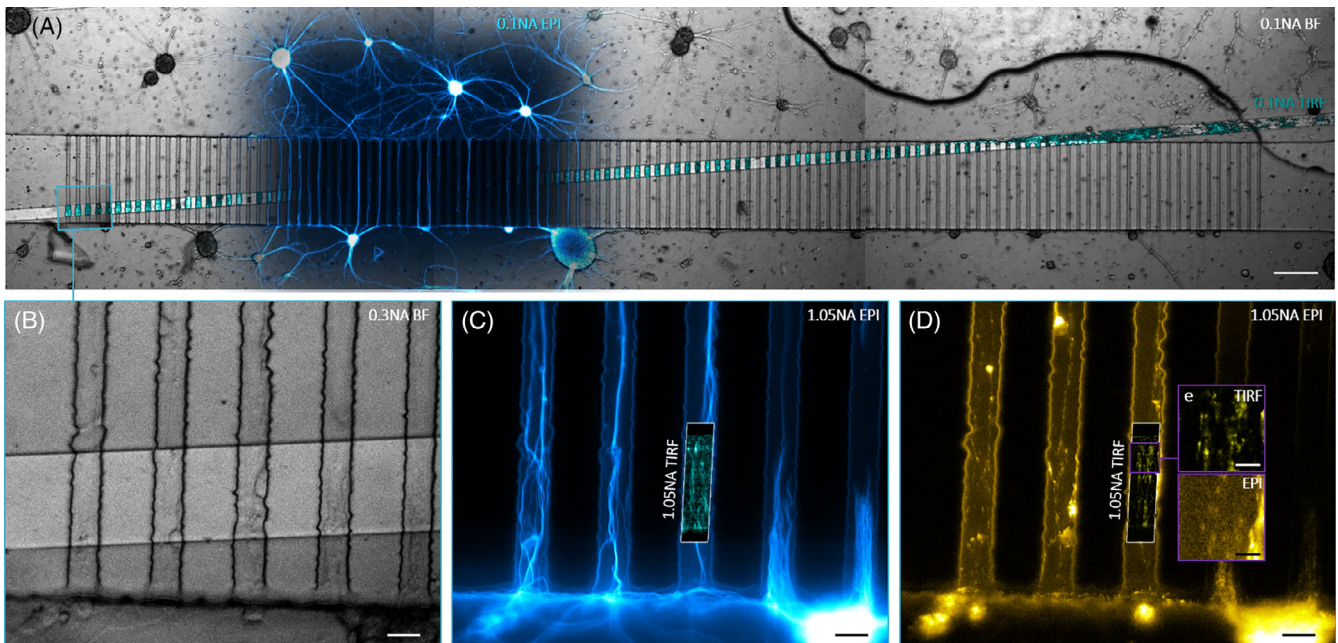

FIGURE 4 Advanced cell culture approaches like microchannel devices can be combined with large FOV chip TIRFM. All panels show rat hippocampal neurons cultured in a chip-microchannel combi device and labelled using SiR-Tubulin and MitoTracker Orange. A, Stitched overview image acquired using a 4× 0.3NA air objective. BF mode overlaid with EPI (blue) and TIRF (cyan) illumination. Scale bar: 200 μm. B-D, Images acquired using a 30× 1.05NA silicon oil immersion objective, scale bars are 20 μm. B, BF image of microchannels on waveguide. C, Corresponding EPI image of microtubules with TIRF image inlay. D, EPI image of MitoTracker Orange with TIRF inlay. E, Zoomed view of TIRF and EPI image of mitochondria in microchannels, scale bar 5 μm. The particular Ta_2O_5 waveguide used for this experiment was 50 μm wide, although any waveguide dimension could be applied in general

Advanced cell culture approaches are becoming more and more important in neuroscience research, such as the microgroove cell culture chambers used in studies of axonal injury or protein transport [15–17]. We therefore sought to use PDMS microgroove chambers in combination with the photonic chip imaging system. One complication is that the cell culture chambers are usually permanently bonded to the substrate, but the chips are—at present—too expensive to be disposed of after a single experiment. We found that clean PDMS was sufficiently tacky to adhere to the photonic chip and to contain media while the neurons grew. Furthermore, as the chips are opaque, signal must be collected from above. This requires the PDMS to be thin enough to match the working distance of the desired imaging objective, and to be uniform and optically clear, so that high quality imaging can be performed through the PDMS layer. However, such a thin PDMS layer would not contain sufficient medium to support the neurons and overcome evaporation. We addressed these challenges by using a second PDMS layer on top of the microfluidic devices to contain the medium, which was removed before imaging, as depicted in Figure 2B, bottom row. As displayed in Figure 4, we successfully performed TIRF, EPI and BF microscopy on photonic chips through PDMS microgrooves of living primary hippocampal neurons using a high NA silicon oil immersion objective. The results show that this multimodal imaging scheme is feasible, although the TIRF imaging success in this case was modest as the axons appeared to have detached from the waveguide TIRF excitation area. This might be addressed in future experiments e.g. by selective coating of the waveguide surface with poly-L-lysine, without coating the microchannel walls, thus removing possible attachment points for the growing axons above the waveguide surface. Another challenge with fluorescence imaging in microgrooves concerns the labelling and consecutive washing steps required in many protocols, which are difficult to achieve in the narrow channels. In our data, this resulted in high background signal from the used MitoTracker label, causing the mitochondria to be barely visible in the EPI image of Figure 4E. Supplementary Note 4 provides details on the procedures and microgroove production steps.

In summary, we have adapted photonic chip large area TIRFM for live-cell neuroimaging applications by developing on-chip cell culture protocols and integrating chip microscopy with a heater system and incubation chamber. We have shown successful cell culture of primary rat hippocampal neurons and explanted *Xenopus* eye primordia and performed large FOV live-cell TIRFM of these sensitive cell types. We have demonstrated imaging on combined microgroove-waveguide devices and the capabilities of our system. We expect the integration of environmental control with the unique advantages of TIRF illumination provided by waveguides to inspire and enable many new imaging applications for photonic chips.

CONFLICT OF INTEREST
CFK received funding from MedImmune | AstraZeneca and Infinitus China Ltd. The other authors declare no competing interests.

AUTHOR CONTRIBUTIONS
I.S.O. led the project and performed the imaging. F.S. designed the ChipScope. I.S.O. and F.S. built and calibrated the system. B.S.A., F.T.D., and J.C.T. provided waveguides and advised on chip microscopy. I.S.O., F.S., and O.V. designed and installed the custom imaging incubator. M.F. wrote the ChipScope control software. C.H. excised, cultured and prepared labelled samples of primary rat hippocampal neurons on chip. C.H. advised on the incubator and helped plan and conduct the imaging experiments. O.V. designed and fabricated the microchannel moulds. I.S.O., C.H., and O.V. made microchannels and PDMS chambers. J.Q.L. and F.W.v.T. provided expertise on *Xenopus* biology and handling. F.W.v.T. performed *Xenopus* dissections and on-chip culturing and advised on the labelling and imaging of RGCs. C.F.K. and B.S.A. provided supervision and funding for the project. G.S.K.S. provided facilities for primary neurons excision and cell culture, and CFK microscopy lab area and equipment. I.S.O. and F.S. wrote the manuscript. All authors contributed to or commented on the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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