sideSPIM – selective plane illumination based on a conventional inverted microscope

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Abstract: Previously described selective plane illumination microscopy techniques typically offset ease of use and sample handling for maximum imaging performance or vice versa. Also, to reduce cost and complexity while maximizing flexibility, it is highly desirable to implement light sheet microscopy such that it can be added to a standard research microscope instead of setting up a dedicated system. We devised a new approach termed sideSPIM that provides uncompromised imaging performance and easy sample handling while, at the same time, offering new applications of plane illumination towards fluidics and high throughput 3D imaging of multiple specimen. Based on an inverted epifluorescence microscope, all of the previous functionality is maintained and modifications to the existing system are kept to a minimum. At the same time, our implementation is able to take full advantage of the speed of the employed sCMOS camera and piezo stage to record data at rates of up to 5 stacks/s. Additionally, sample handling is compatible with established methods and switching magnification to change the field of view from single cells to whole organisms does not require labor intensive adjustments of the system.

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1. Introduction

Selective plane illumination microscopy (SPIM) is one of the most suitable techniques for fast, three-dimensional imaging. By confining the excitation light to a sheet, SPIM combines axial sectioning capability with minimal light exposure and fast, camera-based image acquisition [1]. SPIM typically uses two (objective) lenses arranged perpendicular to each other. One lens is used for light detection, while the focal plane of that lens is illuminated with a sheet of light generated via the other lens. To generate the light sheet, cylindrical optics can be used. Alternatively, the beam can be rapidly scanned across the field of view of the detection lens to generate the sheet illumination. However, the arrangement of two objective lenses perpendicular to each other provides a number of challenges in terms of instrument design and sample geometry.

Initially, as described in [1], SPIM was designed around the specimen with excitation and detection in the horizontal plane. This requires specific sample preparation, typically embedding the sample in a hydrogel such as agarose. This setup excludes the use of conventional sample mounts, such as coverslips, culture dishes and multi well plates. To overcome this limitation, a popular approach is to dip into the sample container from the top, with both lenses typically but not necessarily at a 45° angle with respect to the sample plane. Such a system can be mounted on top of an inverted microscope [2] or implemented as an independent instrument [3]. In this configuration, the objectives are immersed in the same fluid as the sample, which in most cases is either air or water. With water dipping lenses, a numerical aperture (NA) of up to 0.8 can be utilized. Lenses of higher NA can be used by raising the sample into the gap between the two lenses. These large NA lenses are needed for the application of fluorescence fluctuation methods [4]. The drawbacks of this geometry include the requirement of a large sample container to accommodate both lenses resulting in a large immersion volume (~20 ml). This can cause sample disturbance due to flow/convection and increases the amount of reagents needed. Further, there is no isolation of optics and sample which is problematic when dealing with hazardous samples (toxic, cancerous, infectious, etc.). Moreover, dipping into the sample container from the top limits access from that direction. This makes it difficult to fit devices for sample support and monitoring such as incubators, microfluidic devices, electrodes, brightfield illumination, etc. Finally, since the observation plane is at an angle with respect to the sample container, the field of view for flat samples, such as a monolayer of cells, is limited, i.e., the full field of view of the detector cannot be utilized. Another approach to using high NA lenses is reflected light sheet microscopy, in which the light sheet is generated by reflecting a beam incident from the top by 90° with a small mirror mounted on an atomic force microscope cantilever [5]. With this approach, the light sheet is parallel to the sample plane, hence, for flat samples such as a cell monolayer, the full field of view of the detector can be utilized. However, this method requires precise positioning of the mirror very close to the sample. Also, the mirror as well as the excitation lens are introduced from the top and dipped into the sample container, again limiting access and prohibiting sample isolation. Also, chemicals present in the immersion fluid can degrade the mirror so it has to be replaced regularly. Finally, objects very close (~2 µm) to the bottom of the sample container, such as the bottom membrane of a cell, cannot be imaged in this configuration. Alternatively, high NA detection can be realized via a prism-coupled light-sheet condenser design that redirects the light sheet horizontally onto a sample at the focal plane of an imaging objective [6]. The lack of a cantilever facilitates sample handling and operation of the system. However, the sample container is tilted at a horizontal angle of approximately 20°, so care has to be taken when filling the sample dish with the immersion fluid. Again this design demands open access from the top with the same drawbacks as mentioned before. A design that allows access from the top uses a water prism that compensates for aberrations introduced when illumination and imaging from the bottom at an angle through a cover glass [7]. However, this solution cannot be mounted on a regular inverted microscope due to size constraints. Also, this configuration does contain additional
sources of aberration, primarily due to imaging through a tilted cover slip. Hence, it is more suitable for low resolution imaging. Other designs integrate a sample cuvette in the form of a capillary with side illumination into a stage inset of an inverted microscope [8–11]. While this approach is compact and low cost, it provides relatively low axial resolution (~5 µm) and/or demands specific tube-mounted samples. Finally, SPIM implementations using a single lens do not suffer from opto-mechanical constraints of two lens designs but are limited in spatial resolution and/or imaging depth [12–16].

This manuscript describes a new selective plane illumination method in the conventional sample geometry. Our design is based on a regular inverted microscope where the sample is illuminated from the side via an accessory. A custom designed chamber with multiple wells featuring two optically transparent windows is used to allow side illumination and light detection from the bottom. In this design, all microscope ports remain available for other purposes. Also, there is unrestricted access from the top which can be used, for example, to fit the connections of a fluidic device. Without the need of dipping into the sample container, smaller sample volumes (<1 ml) can be realized and the use of high NA lenses is facilitated. Still, all kinds of samples can be used including both, flat samples such as monolayers of cells or bacteria on a surface and specimen such as cells, tissues, and organisms embedded in hydrogels. Distortion-free imaging of flat samples is achieved via matching of the refractive index. Also, isolation of optics and sample allows imaging of sealed sample containers when demanded, e.g., for samples treated with potent toxins. Further, in this design, the orientation of the imaging plane is parallel to the surface of the sample container which is desirable for flat samples where it maximizes the field of view and avoids the sagittal view of the specimen. Finally, since the observation volume can be very small, high throughput 3D imaging of multiple wells is possible by fast and precise stage movement.

2. The sideSPIM concept

The three key components of the sideSPIM system include the side illumination unit, the two window sample chamber, and, if the sample needs to be mounted on a flat surface, a transparent support with the same refractive index as the surrounding medium. Those three components are described in the following.

2.1 Side illumination unit

All optical components required to generate the light sheet illuminating the sample were mounted onto a single platform named the side illumination unit. Notably, this accessory unit can be coupled to any inverted microscope. Our system was based on an IX71 inverted microscope (Olympus, Waltham, MA, USA) fitted with camera detection (Edge 4.2, PCO, Romulus, MI, USA). A motorized xy stage (MS-2000, ASI, Eugene, OR, USA) held a z piezo top plate (PZ-2000, ASI) fitted with a custom magnetic sample holder inset. The stage assembly was raised by 36 mm using spacers to make room for the objective lens of the side illumination unit located on the left hand side of the microscope body. Besides being installed onto the same flat and rigid mounting surface (Smart Table UT2, Newport, Irvine, CA, USA), no further mechanical connections from the side illumination unit to the microscope body were required. A schematic of the side illumination assembly is shown in Fig. 1(A). Within this unit, the light sheet was generated and injected into the sample. The assembly consisted of a white laser source, WL (SC 390, Fianium Ltd, Southampton, UK), for excitation with visible light. From the fiber output the light was reflected off a dichroic mirror, DM1 (T670 LPXR, Chroma, Bellows Falls, VT, USA), and passed through a short pass filter, SP (682/SP HC, Semrock, Rochester, NY, USA), to remove the near IR portion of the laser output which was directed into a beam trap, BT (LB1, Thorlabs, Newton, NJ, USA). The visible portion was passed through a shutter (LS3, Uniblitz, Rochester, NY, USA) followed by a motorized filter wheel, F1-6 (FW102C, Thorlabs), containing six different filters, 440/40 ET, 480/30 ET, 535/30 ET (Chroma), 572/15 BrightLine HC (Semrock), FL632.8-10, and NE30A (Thorlabs),
which defined the excitation wavelength bands. To ensure a Gaussian beam profile, the filter wheel was followed by a spatial filter. In the spatial filter, the laser beam was focused onto a 10 µm pinhole, PH (P10S, Thorlabs), via a lens of 30 mm focal length, L1 (AC254-030-A, Thorlabs), and collimated by a lens of 50 mm focal length, L2 (AC254-050-A, Thorlabs). Redirected with a mirror, M1, the beam was then passed through an adjustable iris, I (SM1D12, Thorlabs), to control the beam diameter. Reflected off a second mirror, M2, and a second long pass dichroic mirror, DM2 (T670 LPXR, Chroma), the beam was redirected onto the scanning mirror assembly, XY (A402, ISS Inc., Champaign, IL, USA). In addition, a pulsed tunable Ti:Sa laser (Chameleon Ultra, Coherent, Santa Clara, CA, USA) for two-photon excitation located behind the sideSPIM setup on the same optical table was free space coupled into the side illumination unit from the bottom. The laser intensity was modulated by an acousto optic modulator (AOM, AA Opto-Electronic, New York, NY, USA) placed immediately after the laser output. After directing the beam to the illumination unit via four mirrors on the optical table it was collimated by a telescope consisting of two lenses of 50 mm focal length, L3 and L4 (AC254-050-B, Thorlabs). Via two more mirrors, M3 and M4, the near infrared beam was passed through the long pass dichroic mirror, DM2 (T670 LPXR, Chroma), to be joined with the visible laser light. The combined beam was then relayed towards the excitation objective, OE (10x CFI Plan Fluorite NA 0.3, Nikon, Melville, NY, USA), via a scan lens, SL (#49-356, Edmund Optics Inc., Barrington, NJ, USA), and a tube lens, TL1 (180 mm, Olympus). We note that two-photon excitation was not used for the work presented in this manuscript, therefore, parameters of the near infrared beam were not further characterized. Rapid scanning of the horizontal axis resulted in the generation of a light sheet in the plane of the detection lens. Alternatively, instead of scanning the beam, cylindrical optics could be used to generate the sheet. The scanning, however, facilitates two-photon excitation and has the advantage that non-Gaussian beam profiles could be generated. The fluorescence light generated in the sample was collected by the detection lens, OD (LUMPLFLN60x/W NA 1.0, Olympus), and imaged onto the CMOS camera (Edge 4.2, PCO) mounted to the left side port of the microscope after passing through the internal fluorescence filters, F7-10, 447/60 BrightLine HC, 535/50 BrightLine HC, 630/69 BrightLine HC, and 647 LP (Semrock), and tube lens, TL2 (180 mm, Olympus). Brightfield illumination was performed via the lamp and condenser arrangement mounted on top. The right side port of the microscope was still available and could be fitted with another excitation/detection system. A photograph of the complete system is shown in Fig. 1(B). The inset of the piezo stage was fitted with a custom sample holder. It consisted of a vertically mounted linear stage (MS1S, Thorlabs) onto which the actual sample holder was attached to. This linear stage allowed for coarse adjustment of the sample z position. The detection lens was located in the turret that was part of the inverted microscope. Hence, detection lenses could be easily changed by rotation of the turret, if different magnifications were required for image acquisition. In the prototype described here, we switched between a 40x NA 0.8 water objective (LUMPLFL40x/W NA 0.8, Olympus) and the 60x NA 1.0 water dipping lens in detection depending on the resolution/field of view demanded. A photograph of the arrangement of excitation and detection lenses and the sample chamber/sample holder is shown in Fig. 1(C). A rubber O-ring was utilized to prevent excess water from flowing down the 60x NA 1.0 water dipping lens. Any combinations of excitation and detection lens can be used, with the only (geometrical) constraint that the focal points of the two lenses have to overlap without collision of the two lenses. The excitation lens was mounted on a xy manual platform (MT1, Thorlabs) attached to the illumination unit to align the light sheet with the optical axis of the detection lens. The microscope turret z drive was then utilized to align the focal plane of the detection lens with the light sheet. Three-dimensional imaging was achieved by scanning the sample in axial direction with the z piezo top plate. The sample holder was machined from aluminum, its t-shaped legs held four magnets in each corner to ensure easy and secure sample attachment. The screws that attached the legs to the frame
acted as pins that fit into corresponding holes (5 mm diameter) in the sample chamber for reproducible sample placement.

Fig. 1. Illustration of the sideSPIM system and the two window sample chamber. (A) Schematic of the optical components involved in the side illumination unit (see main text for a detailed description). (B) Photograph of the sideSPIM system. (C) Photograph of the sample chamber mounted in the sideSPIM setup showing the organization of excitation and emission lenses. (D) 3D drawing of the two window sample chamber. (E) Photograph of the finished two window chamber.
A common problem when imaging through a cover slip with high NA lenses are aberrations caused by a tip/tilt of the glass surface with respect to the focal plane of the objective lens. Our system showed a tip of 0.29° and a tilt of 0.15° of the cover slip with respect to the focal plane of the detection lens (NA 1.0). For a NA 1.2 water immersion lens the maximum tip/tilt acceptable for distortion free imaging was reported as 0.3° [17]. Hence, our system did not require further alignment. Nevertheless, as in any other microscope setup using high NA lenses, it might be useful to integrate a system to allow for tip/tilt adjustment.

2.2 Two window sample chamber

With two optically transparent windows perpendicular to each other, the light to generate the sheet illumination at the sample plane can be introduced from the side. Magnetic attachment of the chamber to the microscope stage ensured easy to handle, stable and reproducible mounting. In order to inject the light sheet into the sample from the side with the side illumination unit, the sample chamber needs to have two optically transparent windows, one on the bottom and one on the side of each well. A sketch of our sample chamber design is shown in Fig. 1(D). We laser cut (Epilog Fusion Laser Cutter, UCI Fabworks, Irvine, CA, USA) the backbone of the chamber from ¼” thick acrylic sheet (8560K358, McMaster, Santa Fe Springs, CA, USA). Three pieces of glass were then attached to create the chamber wells, one on the bottom and two on the sides of the chamber backbone. For the bottom, we used commercially available cover glass measuring 60 mm × 24 mm of 0.17 mm thickness (22266882, Fisherbrand, Thermo Fisher Scientific, Huntington Beach, CA, USA). The two side windows (52 mm x 6 mm) were cut from the same cover glass slides using an engraving pen (Z225568-1EA, Sigma-Aldrich, St. Louis, MO, USA). Watertight attachment was achieved by means of an UV curable optical adhesive (NOA60, Thorlabs). Finally, four steel foils were glued on the corners to allow the chamber to attach to the four magnets embedded in the feet of the sample holder. A photograph of the fully assembled chamber is shown in Fig. 1(E). The exact dimensions of the sample wells are not critical for the function of the system and can be adapted to optimally accommodate the sample under study. Especially, since the well size can be very small, a large number of wells can be arranged in a line to allow for high throughput imaging. The application of SPIM for high throughput imaging was previously proposed by the use of a fluorinated ethylene propylene (FEP) tube though which the sample can pass via pump aspiration. However, in this configuration the spatial resolution is limited due to the use of low NA objectives [18]. In the design presented in this work, we can acquire images with high NA objectives on the emission side. Additionally, the cell media can be modified independently for each well during the experiment with a fluidic approach. For imaging, each well can be addressed in an automated way by movement of the sample chamber with the motorized stage. The only requirements of our design are two thin, optically transparent windows, one on the bottom and one on the side. For the prototype we used microscope cover glass of 0.17 mm thickness but other transparent materials would work as well. For samples embedded in a transparent medium there are no further requirements for imaging. It has to be noted, however, that as soon as a significant amount of light passes through a material of different refractive index, e.g., when trying to image an object close to the bottom of the sample chamber, optical aberrations occur. This can be avoided by matching of the refractive index, which is discussed in the following.

2.3 Refractive index matching

While in solution or in a hydrogel (e.g. agarose, collagen, gelatin, etc.) the mismatch in refractive index between the bottom window of the chamber and the sample mounting medium can be avoided by imaging in the center of the mounting medium (see Fig. 2(A)), imaging a monolayer of cells or bacteria on a surface represents exactly such problem (Fig. 2(B)).
Fig. 2. Refractive index matching. (A) Inside a solution or hydrogel, a sample can be imaged without further modifications. (B) Close to the bottom of the chamber, a mismatch in refractive index occurs distorting the light sheet. (C) A distortion of the light sheet can be avoided by raising the sample on a support with a refractive index similar to the surrounding medium. (D) Simplified light path inside the chamber without (top) and with a mismatch in refractive index (bottom), the insets show the corresponding diffraction patterns at the focus. (E) Graph of the intensity profile of the beam at the focus along x direction for index mismatches of 0-0.5% (NA 0.3, 1 mm depth, 500 nm light). (F) Beam waist (e−2) at the focus and Strehl ratio plotted as a function of the refractive index mismatch, the inset shows the diffraction pattern at 0.3% mismatch, the maximum intensity has shifted from the focus to the periphery of the light sheet as indicated by the arrows.

However, a mismatch can be avoided if the flat sample is mounted on top of an optically transparent material with a refractive index that is similar to the surrounding medium (Fig. 2(C)). Hence, by raising the sample inside the chamber well using an optically transparent material with a refractive index identical to the sample immersion fluid, samples can be imaged distortion free all the way to the bottom. Thus, index matching allows imaging of flat samples such as a monolayer of cells. The maximum allowed deviation from the index of the immersion medium mainly depends on the NA of the excitation lens and the distance from
the side window. The excitation light path in the sample chamber can be simplified as illustrated in Fig. 2(D). A lens with focal length, \( f \), and aperture, \( d_o = 2r_o \), is focusing the excitation beam in a transparent medium which has a refractive index \( n_{sl}(x) = n_1 \) above and refractive index \( n_{sl}(x) = n_2 \) below the optical axis. The incident light wave, \( U_0(x, y) \), is assumed to be a monochromatic plane wave of unity amplitude propagating along the \( z \) axis. The lens and the following medium introduce a phase retardation, \( \phi(x, y) \), proportional to the local thickness of the lens, \( \Delta(x, y) \),

\[
\phi(x, y) = k n_L \Delta(x, y) + k n_{sl}(x) \left[ \Delta_0 - \Delta(x, y) \right],
\]

with refractive index of the lens, \( n_L \), maximum thickness of the lens, \( \Delta_0 \), and wave vector \( k \). Hence the field leaving the lens, \( U_L(x, y) \), becomes

\[
U_L(x, y) = t_0(x, y) e^{-i k \Delta_0 (x)} e^{-i \left[ n_L - n_{sl}(x) \right] \Delta(x, y)},
\]

where the transmission by the lens aperture is \( t_0(x, y) = 1 \) for \( x^2 + y^2 \leq r_o^2 \) and \( t_0(x, y) = 0 \) for all other values. In paraxial approximation, the local thickness of the lens can be described by

\[
\Delta(x, y) = \Delta_0 - \frac{x^2 + y^2}{2} \left( \frac{1}{R_1} - \frac{1}{R_2} \right),
\]

with \( R_1 \) and \( R_2 \) the curvature of the lens faces which can be substituted by the focal length

\[
\frac{1}{f(x)} = \left[ n_L - n_{sl}(x) \right] \left( \frac{1}{R_1} - \frac{1}{R_2} \right).
\]

Substitution of Eqs. (3), (4) in Eq. (2) yields the field immediately behind the lens

\[
U_L(x, y) = t_0(x, y) \exp(-i k \Delta_0 n_L) \exp \left( i k \frac{x^2 + y^2}{2 f(x)} \right).
\]

This field further propagates along the optical axis, Fresnel diffraction can be used to calculate the field, \( U_z(x_z, y_z) \), at a distance \( z = f \),

\[
U_z(x_z, y_z) = -\frac{\exp(-i k z)}{i \lambda z} \exp\left( -i k \frac{x_z^2 + y_z^2}{2z} \right)
\]

\[
\cdot \int_{-\infty}^{\infty} dx \int_{-\infty}^{\infty} dy U_L(x, y) \exp\left( -i k \frac{x^2 + y^2}{2z} \right) \exp\left( -i k \frac{x y}{z} \right),
\]

where \( \lambda \) is the wavelength of the excitation light with \( |k| = 2\pi / \lambda \). After substituting \( U_L(x, y) \) in Eq. (6) the field at the focal plane \( z = f \) of the lens becomes
To estimate how closely the index of immersion medium, \( n_{im} = n_1 \), and mounting medium, \( n_{mt} = n_2 \), has to match we calculated the diffraction pattern by numerical evaluation of Eq. (7) assuming an excitation wavelength of 500 nm, an excitation lens NA of 0.3, and an imaging depth of 1 mm from the side (computation was done in Matlab R2016b, MathWorks, Natick, MA, USA). The main effects of the index mismatch are a translation of the excitation beam focus along the optical axis, a shift normal to the interface of immersion medium and mounting medium, a broadening in width and a decrease in amplitude (see Fig. 2(D), insets). The displacement of the beam focus can be easily compensated by adjusting the position of the excitation beam. Therefore, we focused on the increase in beam waist and decrease in amplitude as a criterion for the tolerable index mismatch. Figure 2(E) shows a graph where the cross section of the intensity profile along the \( x \) axis at the focus is plotted as a function of the refractive index mismatch, \( 1/n - n_2 \), in percent. The increase in the beam waist (\( e^{-2} \)) was quantified in Fig. 2(F) by fitting a Gaussian distribution to the intensity profiles plotted in Fig. 2(E). We further characterized the decrease in intensity by calculating the Strehl ratio, which is the ratio of the peak intensity of the aberrated beam (here, \( \Delta n > 0 \)) to the maximum intensity of the ideal beam (here, \( \Delta n = 0 \)), a common criterion to quantify aberrations in optical systems. Although the Strehl ratio exhibits a rapid decrease, the minimum beam width remains almost constant until a mismatch of around 0.4%. While the intensity at the beam focus decreases we saw an increase in the intensity profile towards the periphery of the beam (see Fig. 2(F), inset). This would actually be beneficial since it would increase the confocal parameter, i.e., increase the beam portion that is reasonably thin. However, with a further increase of the index mismatch the beam exhibits a curvature and the beam periphery will no longer overlap with the focal plane of the detection lens. Therefore, with an excitation NA of 0.3, as used in our system, this calculation suggests that the mismatch in refractive index should ideally be <0.2%. Such material is commercially available, e.g., in the form of an UV curable resin (MY-133/MY-133 V2000/MY-134, EOC-Inc, Santa Rosa, CA, USA). We cured the resin between two glass slides to achieve a flat surface with a thickness of 1 mm. After curing, the resin was cut to size and transferred into the sample chamber. It has to be noted that this particular resin is very hydrophobic and, depending on the specimen to be imaged, surface coating/modification may be required. Alternatively, hydrogels such as collagen also have a refractive index very close to water and can be used as substrate as well. A brief overview of the refractive index of common immersion fluids and a selection of suitable mounting materials is shown in Table 1.

Biological samples, especially live cells, are typically immersed in a specific medium containing salts and nutrients (e.g. DMEM – Dulbecco’s Modified Eagle Medium). Hence the refractive index to be matched is slightly higher than the index of pure water.

\[
U_i(x, y) = -\frac{\exp(-ik)}{i\lambda z} \exp\left(-ik\frac{x^2 + y^2}{2z}\right) \exp(-ik\Delta_n n_2) \cdot \int dx \int dy \exp\left(\frac{ik}{2f(x)}\frac{x^2 + y^2}{2f(x)}\right) \exp(-ik\frac{xx + yy}{z}).
\]
Table 1. Refractive Index of Common Compounds

| Compound          | n (at 589 nm) ± SD | %Δn water |
|-------------------|-------------------|-----------|
| Water             | 1.3330            | -         |
| PBS 1x            | 1.3347 ± 0.0002   | 0.13      |
| DMEM + F12        | 1.3359 ± 0.0002   | 0.21      |
| MY-133            | 1.3371 ± 0.0003   | 0.31      |
| MY-133 v2000      | 1.3342 ± 0.0001   | 0.10      |
| Collagen 2 mg/ml  | 1.3357 ± 0.0002   | 0.20      |
| Agarose 1%        | 1.3343 ± 0.0001   | 0.10      |

List of compounds with their refractive index measured with an Abbe Mark II Refractometer (Reichert, Depew, NY, USA) and difference with respect to water (each value represents the average of 15 measurements, the error stated is the standard deviation).

3. Results

To validate and illustrate the capabilities and versatility of the sideSPIM approach, we imaged a wide variety of samples from the micrometer to the millimeter scale including fluorescent beads, giant unilamellar vesicles, mammalian cells in 2D and 3D cell culture, bacteria composing a biofilm, and a zebrafish embryo.

3.1 Imaging with index matching

To test the sideSPIM system, we first imaged a 100 nM solution of Rhodamine 110 in water (Milli-Q, EMD Millipore, Billerica, MA, USA). Fluorescence was excited in a band of 480/30 nm (100 μW before the excitation objective) and detected through a 535/50 nm band pass filter with a 60x NA 1.0 detection lens. Without scanning the beam along the x direction (amplitude of the scanner 0 V) the extension of the light sheet in the plane of excitation was defined by the excitation lens NA of 0.3. A sketch of the experiment is shown in Fig. 3(A) the xy image of the fluorescence signal from the Rhodamine 110 solution can be seen in Fig. 3(B). For each vertical line of pixels a Gaussian was fitted to the intensity distribution, the resulting beam waist (distance from the distribution maximum at which the intensity has fallen to $e^{-2}$) as a function of the distance from the focus is plotted in Fig. 3(C). The minimum thickness (maximum axial resolution) was found to be 1.43 μm while the confocal parameter (two times the Rayleigh length, $z_R$) was 12.0 μm. To verify that optical aberrations are minimal after introducing the resin to match the refractive index, we placed a 10 × 10 mm² piece of 1 mm thick resin (MY-133 V2000) into a sample chamber well and filled it with the same 100 nM solution of Rhodamine 110. We took an image with the excitation beam at a distance of 1 μm from the resin/Rhodamine solution interface (as depicted in Fig. 3(D)), the corresponding intensity distribution is shown in Fig. 3(E), the beam waist as a function of the distance to its minimum is plotted in Fig. 3(F). The minimum extension was 1.53 μm, the confocal parameter was 10.9 μm. Another image was acquired right at the resin/Rhodamine solution interface (Fig. 3(G)-3(I)), there the minimum extension was 1.49 μm, and the confocal parameter was 11.8 μm. While, in a homogeneous solution, the shape of the beam should be identical in the xy plane compared to the xz plane, this could be different after introduction of the resin which acts as a divider in axial direction. Therefore, we acquired a z stack while scanning the beam in x direction to illuminate the whole field of view near the resin/Rhodamine solution interface (as depicted in Fig. 3(J)), a single xz plane is shown in Fig. 3(K). The beam shape in xz direction was obtained by fitting a Gaussian to the derivative of the intensity distribution in each vertical line (Fig. 3(L)), the minimum extension was 1.46 μm, the confocal parameter was 11.0 μm. All fluorescence images of Rhodamine 110 in solution were subjected to deconvolution with the detection point spread function (PSF) using the Lucy-Richardson method (deconvlucy, Matlab R2016b). The detection PSF was modeled.
as a Gaussian with 0.37 µm width as experimentally determined from images of 100 nm green fluorescent beads (see next paragraph).

Fig. 3. (A-L) Light sheet characterization with and without index matching in a 100 nM solution of Rhodamine 110. (B) Fluorescence image of a single xy plane inside the Rhodamine solution without scanning and no resin in the sample well as illustrated in (A). (C) For each vertical line of pixels, a Gaussian was fitted to the intensity distribution, the minimum beam waist was 1.43 µm, the confocal parameter was 12.0 µm. (E) Fluorescence image of a single xy plane inside the Rhodamine solution at a distance of 1 µm from the resin (MY-133 V2000) without scanning as illustrated in (D). (F) The minimum beam waist was 1.53 µm, the confocal parameter was 10.9 µm. (H) Fluorescence image of a single xy plane at the Rhodamine solution/resin interface without scanning as illustrated in (G). (I) The minimum beam waist was 1.49 µm, the confocal parameter was 11.8 µm. (K) Fluorescence image of a single xy plane extracted from a z stack of the Rhodamine solution/resin interface as illustrated in (J). (L) The minimum beam waist calculated from the intensity derivative was 1.46 µm, the confocal parameter was 11.0 µm. (M-X) Light sheet characterization using 100 nm green fluorescent beads. Single xy (M) and yz (N) plane of a stack of fluorescence images of 100 nm beads embedded in a 1% agarose hydrogel. The xy (O) and yz (Q) cross sections of the PSF of an exemplary bead (marked by the crosshair in M,N) were fitted with a Gaussian distribution (P,R) to obtain the radial and the axial waist. Single xy (S) and yz (T) plane of a stack of fluorescence images of 100 nm beads embedded in a 1% agarose hydrogel placed on top of the 1 mm thick resin (MY-133 V2000). The xy (U) and yz (W) cross sections of the PSF of a representative bead (marked by the crosshair in S,T) were fitted with a Gaussian distribution (V,X) to obtain the radial and axial waist.
To further evaluate imaging performance of the sideSPIM setup, we prepared a sample with green fluorescent spheres of 100 nm diameter (yellow-green Fluorospheres, Invitrogen, Thermo Fisher Scientific) dispersed in a 1% agarose hydrogel. Three dimensional stacks were acquired, single xy and yz planes are shown in Fig. 3(M), 3(N). Zoomed-in xy and yz images of the bead marked by the crosshairs and plots of the corresponding cross sections in y and z direction are presented in Fig. 3(O)-3(R). We measured a total of 10 beads, the average radial waist was 0.37 ± 0.02 µm while the average axial waist was 1.2 ± 0.2 µm (mean ± standard deviation, SD). Next, the resin was placed in the sample chamber and topped with the same hydrogel containing 100 nm fluorescent beads. Single xy and yz planes of a z stack are shown in Fig. 3(S), 3(T). It can be seen that the beads are clearly visible all the way down to the surface of the resin. Zoomed-in xy and yz images of a bead at 5 µm distance from the resin/hydrogel interface as indicated by the crosshairs and plots of the corresponding cross sections in y and z direction are presented in Fig. 3(U)-3(X). The average radial and axial waist of several beads at a distance of 0-5 µm from the resin/hydrogel interface was found to be 0.39 ± 0.02 µm and 1.5 ± 0.1 µm (N = 10, mean ± SD). Hence, there is a slight but negligible loss in spatial resolution introduced by the resin. A common issue with light sheet microscopy is that if the light sheet is formed with a Gaussian beam, the axial resolution diminishes towards the periphery of the light sheet (i.e., if the field of view extends beyond the confocal parameter). This loss in axial resolution could be avoided by using non-Gaussian beams [19] or by scanning the light sheet in axial direction [20–22].

3.2 Three-dimensional imaging of GUVs on top of a transparent support

Various samples were prepared to demonstrate that objects can be imaged without significant distortion on a transparent support of a refractive index very close to the index of the sample immersion fluid. Giant unilamellar vesicles (GUVs) are a popular in vitro model for studying lipid membrane dynamics as a function of lipid composition as well as protein-lipid interactions [23]. However, these vesicles are very difficult to image with most SPIM configurations. GUVs cannot be mounted in a hydrogel, the supporting surface must be level to avoid accumulation of the vesicles at the point of lowest elevation and slight changes in osmolarity, for example due to contamination of the immersion fluid by dipping into the sample chamber with a lens, lead to bursting of the GUVs. Additionally, it is highly desirable to work with a small sample volume (<1 ml) to save reagents and facilitate GUV formation. We prepared fluorescently labeled GUVs with different lipid compositions. To attach the GUVs to the resin (MY-133 V2000) surface we used a coating protocol with biotin-bovine serum albumin (b-BSA). Briefly, the resin was coated with a solution of 1% BSA and 0.1% b-BSA. The GUVs were doped with 0.1 mol % of biotin-phosphatidylethanolamine (b-PE) and 0.5 mol % of DiIC18 (D3911, Invitrogen). An electro-formation protocol was used to grow the GUVs; lipids were deposited on a platinum wire and later dried in vacuum for one hour [24]. Then, the Pt wires were connected to a function generator generating a 10 Hz sinusoidal wave with an amplitude of 2 Vp-p for 1 hour. During that time the chamber used for GUV formation was heated above the lipid melting transition (65°C). Then the GUVs were detached from the wire by decreasing the frequency to 1 Hz for 10 minutes. After detaching, the sample was allowed to cool to room temperature. The GUVs were carefully transferred to the chamber containing the coated resin and immediately imaged. Fluorescence of DiIC18 was excited in a band of 572/15 nm and detected using the 60x NA 1.0 objective through a 630/69 nm band pass filter. We acquired z stacks with an axial step size of 500 nm while the camera pixel size at the sample was 108 nm. The differences in lipid composition are immediately apparent from the 3D reconstructions (Figs. 4(A), 4(B)).
While DOPC forms a homogeneous membrane (A), the ternary mixture of DOPC, DPPC, and Cholesterol allows liquid order/liquid disorder (Lo/Ld) phase coexistence (B). Since the dye DiIC18 has different affinities for the Lo/Ld phases, these can be identified as dark patches. Taking advantage of the z piezo stage and the fast camera, we were able to follow diffusion of lipid domains in 3D on the entire GUV as shown in Visualization 1. We were able to image 3D stacks of 60 planes at 1.25 stacks/s. The exposure time for a single plane was 10 ms resulting in 600 ms for all 60 planes plus a 200 ms long overhead for repositioning of the piezo stage at the starting position. The domains freely diffuse on the surface of the GUV, six exemplary tracks are shown in Visualization 1. During the acquisition of the 50 stacks shown, the photobleaching was negligible.

3.3 Three-dimensional imaging of cells on top of a transparent support

Although more and more biological/biomedical studies are utilizing 3D cell culture instead of cells growing on a surface due to the higher physiological relevance of a three-dimensional environment, many fundamental mechanisms can still be unraveled using 2D cell culture.
Therefore, it is important to show that the sideSPIM is compatible with classical cell culture protocols. As noted earlier, the resins used in this work are very hydrophobic and cells typically do not attach without further surface treatment/modification. We utilized a protocol established to grow cells on top of PDMS [25]. Briefly, a polyelectrolyte multilayer (PEM) film was built on top of the resin (MY-134) by alternate adsorption of poly-anions and polycations. Respectively, solutions of poly(diallyldimethylammonium chloride) (PDAC) and sulfonated poly(styrene) (SPS) at concentrations of 0.02 M and 0.01 M (based on the repeating unit molecular weight) were prepared with deionized water with the addition of 0.1 M sodium chloride. The resin surface was placed in a plasma cleaner for 3 min at 0.15 torr. Immediately after surface activation, the resin was immersed in the poly-cation solution for 20 min. After two sets of 5-min long rinses the resin was transferred to the poly-anion solution for 20 min followed again by rinsing twice for 5 min each. Between each deposition of a poly-cation/poly-anion layer the sample was cleaned for 3 min in an ultrasonic bath. After depositing a total of ten bilayers, the resin was placed into a well of a two window sample chamber, followed by coating with fibronectin. CHO-K1 cells stably expressing EGFP were plated in the well and subjected to sideSPIM imaging after incubation for 24h. A single 3D reconstruction of a CHO-K1 cell is shown in Fig. 4(C). A time series of a CHO-K1 cell was recorded as well, stacks of 80 planes with 500 nm z spacing were acquired at time intervals of 1 min, the movie is included as Visualization 2. We note that, even after coating, adverse effects of the resin surface cannot be excluded, especially for more sensitive cell lines. Therefore, it might be more suitable to plate live specimen such as cells on a support of biological material such as collagen, gelatin, agarose or other extracellular matrix hydrogels. As an example, we placed a 1 mm thick layer of agarose inside the well of a two window chamber followed by the addition by the addition of a solution containing \textit{Pseudomonas aeruginosa} AFS64 bacteria expressing EGFP. A stack of 20 planes (500 nm z spacing) was acquired. The 3D reconstruction is shown in Fig. 4D. The sample chamber used for this particular experiment had been modified to include an inlet on one end and an outlet on the opposing end of the well such that it could be used as a fluidic device. As a test, we flowed 1 µm red fluorescent beads through the channel at flow rates of 1–5 µl/min and recorded z stacks consisting of 40 planes each with 500 nm step size at a rate of 3.3 stacks/s, a movie of the corresponding z projections is shown in Visualization 3. A movie of the biofilm under a flow of 5 µl/min is displayed in Visualization 4. Here 3D stacks of 20 planes each (500 nm z spacing) were taken at a rate of 1 stacks/s. It can be seen that the biofilm is very dynamic, bacteria continuously detach from and join the film, especially at the edges. Again this is an example of an experiment particularly difficult to do with most other SPIM configurations. By dipping into the sample chamber it is almost impossible to perform an experiment involving fluidics, let alone microfluidics, while this is not a problem at all with the two window well chamber and the sideSPIM configuration. Wild-type \textit{Pseudomonas aeruginosa} expressing GFP (AFS64) [26, 27] were grown at 37°C overnight to saturation in a roller drum. The overnight culture was back-diluted 1:100, and grown at 37°C in a roller drum (TC-7, New Brunswick Scientific Co., Edison, NJ, USA) to an optical density at 600 nm (OD600) between 0.1 to 0.2. Then, 1 ml of the cultures was transferred to the imaging chamber, previously prepared with a layer of agarose hydrogel, with fresh LB media (LB Broth, BD Bioscience, San Diego, CA, USA) and grown for an additional 2 hrs at 37°C to promote biofilm growth and adhesion. All samples were imaged at room temperature (23°C). For image acquisition the open source microscopy software Micro Manager [28] (https://micro-manager.org/) was used. 3D images were rendered with the 3D viewer which is part of Fiji ImageJ (https://fiji.sc/).

### 3.4 Live cell imaging of samples embedded in a collagen matrix

Recently, the limitations of 2D cell culture experiments have become more and more apparent, especially in the screening of new drugs [29]. There is a paradigm shift towards 3D cell culture models to close the gap between isolated cells on a surface and whole animal...
models. While experiments with cells in a dish are cost-effective and can be done in a high throughput manner, the cells are detached from their biological niche excluding many important stimuli only present on the systems level. Whole animal studies, on the other hand, are highly relevant but slow, costly, and involve many parameters that are difficult to control. 3D cell culture can provide the same cost efficiency, high throughput, and control of experimental parameters as 2D culture but with more physiological relevance. And, in terms of fluorescence data acquisition, SPIM is the ideal tool for fast, three-dimensional imaging with minimal light exposure.

With the high rate of stacks the z piezo stage can acquire, highly dynamic structures are of particular interest. Therefore, we prepared A549 stably expressing ABCA3-EGFP (ATP-binding cassette transporter) in a collagen matrix [30, 31]. Following the manufacturer directions, cells were plated in a mixture of 2 mg/ml collagen type I (BD Bioscience). After two days, the sample was subjected to sideSPIM imaging, fluorescence was excited in a band of 480/30 nm and detected through a 535/50 nm band pass filter. A 3D rendering of a cluster of cells is displayed in Fig. 5(A), a time series of a single cell with stacks of 50 planes (500 nm spacing) acquired every 3.2 s is presented in Visualization 5. This time resolution was sufficient to resolve the movement of the lamellar bodies. Mitochondria are another example of highly dynamic structures in a cell, fusion and fission occurs on the order of seconds [32]. On a standard confocal microscope, this time resolution restricts imaging to a single plane. To demonstrate that our system is capable of capturing those events in 3D, A549 cells were embedded in a collagen matrix and the mitochondria were fluorescently labeled by incubating with TMRE (T669, Thermo Fisher Scientific) at a final concentration of 200 nM for 30 min at 37°C, right before subjecting the sample to sideSPIM imaging. Fluorescence was excited in a band of 572/15 nm and detected through a 630/69 nm band pass filter, 60 planes at 500 nm spacing were acquired. The resulting fluorescence image stack is rendered in Fig. 5(B). A 3D time series of the mitochondria of two adjacent cells was acquired at 4.2 s intervals. The movie of the 3D reconstructions is shown in Visualization 6. Clearly, the time resolution was sufficient to follow mitochondria dynamics not in a single plane but the entire cell instead. We note that collagen is highly scattering, in fact, it is possible to image collagen fibers by collecting scattered excitation light after removal of the fluorescence filter from the detection path. Still, we were able to image fluorescently labeled mitochondria, lysosomes, and lamellar bodies in single cells growing in a collagen substrate without significant aberrations. Yet, hydrogels such as agarose or gelatin might be more suitable for other applications.
Fig. 5. Cells in collagen hydrogels. (A) 3D reconstruction of an image stack of A549 cells expressing ABCA3-EGFP. (B) 3D rendering of a stack of fluorescence images of the mitochondria of an A549 cell labeled with TMRE. (C) 3D reconstruction of an image stack of lysosomes in A549 cells labeled with Lysotracker Red. (D) 3D trajectories of the lysosomes shown in panel C followed over 2,100 s that could be followed in a minimum of 50 consecutive stacks. (E) MSD of those tracks with a velocity >0 µm s⁻¹. (F) Histogram of the velocities of the tracks shown in panel E.
3.5 Three-dimensional tracking of lysosomes in A549 cells

Lysosomes are small vesicles containing enzymes able to digest biomolecules. Besides breaking down polymers, lysosomes also play an important role in cellular processes such as signaling, secretion, repair of the plasma membrane and metabolism [33]. To follow lysosome dynamics in 3D, A549 cells embedded in a collagen matrix were labeled with LysoTracker Red (L7528, Thermo Fisher Scientific) by incubating with a final concentration of 50 nM for 1 h at 37°C immediately before subjecting them to SPIM imaging. Fluorescence was excited in a band of 572/15 nm and detected through a 630/69 nm band pass filter, the 3D reconstruction of a single stack is shown in Fig. 5(C). A series of 500 stacks (60 planes each with 500 nm z spacing) was taken at 4.2 s intervals for a total of 2,100 s. (see Visualization 7, left: z projection, right: x projection) and subjected to 3D particle tracking analysis. Sample drift was compensated by subtraction of the average displacement of all lysosomes detected. Tracks of those lysosomes that could be followed for a minimum of 20 consecutive stacks were included in the data set for further analysis. The tracks are visualized in Fig. 5(D). For visualization purpose a minimum track length threshold of 50 consecutive stacks was applied. All tracks were fitted with a second order polynomial. The mean square displacement (MSD) of those tracks with velocities >0 µms⁻¹ are plotted in Fig. 5(E). A histogram of the velocities found is shown in Fig. 5(F). There seem to be at least two populations of velocities, the first starting from 0 µms⁻¹, the second centered around 0.25 µms⁻¹. It has been shown that lysosome trajectories exhibit periods of long-range transport at median speeds of ~0.6 µms⁻¹ interrupted by periods of diffusion [34]. In our analysis, the MSD for each lysosome was calculated for the entire track length. Hence only an average speed is obtained. The data could be analyzed on a subtrajectory level to obtain a more detailed picture. Typically, the trajectory is thresholded for active transport by defining periods of directed motion as motion in a single direction for a certain amount of time. For particle tracking the 2D/3D particle tracker [35] was used which is part of the MOSAIC ImageJ plugin (http://mosaic.mpicbg.de/ParticleTracker/). MSDs were calculated with a custom script written in Matlab (Mathworks, Natick, MA, USA).

3.6 Zebrafish embryo

A 36 hours post fertilization (hpf) zebrafish embryo was fluorescently labeled by incubating for 12 hours with a zebrafish medium that contained 1 µM of the dye Nile Red. Prior to the addition of the dye the embryo was dechorionated and placed in an incubator at 28°C. For imaging, the embryo was mounted in a well of our two window chamber using a 1.5% solution of agarose (low melting temperature, Sigma-Aldrich) at pH 7. Anesthesia with 0.003% tricaine (3-amino benzoic acid ethyl ester; Sigma-Aldrich) was supplemented to prevent the fish from moving [36]. The sideSPIM needs only minimal adjustments to switch between high spatial resolution imaging of single cells and imaging of whole organisms with a large field of view. Basically, a 4x 0.1 NA objective (PLN4X, Olympus) was placed at the excitation side and the detection lens turret was switched to the next position containing a 40x 0.8 NA water objective (LUMPLFL40x/W NA 0.8, Olympus). No other modifications in the excitation or emission paths were required. We characterized the light sheet generated by the 4x NA 0.1 excitation lens with the same 100 nM solution of Rhodamine 110 as used for characterization of the 10x NA 0.3 lens, the minimum beam waist was determined as 2.5 µm while the confocal parameter was 102 µm. Figures 6(A)-6(C) shows an orthogonal view of a 3D stack of the fluorescently labeled 36 hpf zebrafish embryo. Fluorescence was excited in a band of 572/15 nm and detected through a 630/69 nm band pass filter. It is possible to recognize structures such as the notochord, neural tube, and dorsal aorta. The dark lines are related to structures that absorb the excitation light resulting in reduced excitation of the dye below those points. In the 3D reconstitution presented in Visualization 8 showing a z stack (1 µm spacing) of the tail section of the embryo, it is possible to estimate the resolution obtained.
by the sideSPIM that allows identification of single cells. Then, to demonstrate the high speed of our instrument for fast 3D data acquisition we recorded the microcirculation of erythrocytes in the capillary of the zebrafish embryo (see Fig. 6(D) for a single section). Data was acquired at 5 stacks/s of 40 planes each (1 µm z spacing), the camera frame rate was 200 frames/s. The 3D projection shown in Visualization 9 visualizes the characteristic non-pulsed flow of the microcirculation. In principle, it would be possible to track and measure the flow and speed of every single erythrocyte with a simple tracking approach.

![Fluorescence images of a zebrafish embryo labeled with Nile Red.](image)

**Fig. 6.** Fluorescence images of a zebrafish embryo labeled with Nile Red. (A-C) Single sections of an image stack of the tail section of a 36 hpf embryo. (A) xy view, (B) yz view, (C) xz view, the yellow lines indicate the position of the corresponding views. (D) Single section of an area with microcirculation, the track of a single erythrocyte is shown in red.

### 4. Discussion

By using the sideSPIM we were able to image thick samples on the millimeter scale such as a zebrafish embryo as well as micrometer-sized objects such as single bacteria on the same microscopy platform without labor intensive adjustments. The piezo stage employed was able to record data at up to 5 stacks/s (40 planes, camera frame rate 200 fps), fast enough to capture the microcirculation of erythrocytes in a zebrafish embryo. Surface-mount specimens could be imaged distortion-free by mounting them on a transparent support of a refractive...
index similar to the surrounding medium. No dipping into the sample container was required. The container can be sealed if desired or the space can be utilized for sample support, treatment or additional monitoring. As an example, we converted one of our sample chambers to a fluidic device to look at biofilm dynamics under flow. The introduction of fluidics would be very challenging with SPIM designs based on dipping into the sample container. Also, high numerical aperture lenses can be used with this design resulting in single molecule sensitivity. This allows for the application of methods involving single particle localization and tracking as well as fluorescence fluctuation techniques. The sample volume can be as large or small as desired. Further, the observation plane is parallel to the sample surface maximizing field of view for flat samples and accommodating samples that are sensitive to tilt. Since the side illumination unit as well as the two-window sample chamber are both additions independent of the main microscope platform, SPIM capability can be added to any existing inverted microscope. Additionally, since the size of the individual wells can be very small as opposed to designs that require optics dipping into the sample chamber, a large number of wells can be accommodated within the same chamber to allow for automated, high throughput three-dimensional time course imaging with sideSPIM. We believe our implementation will bring more researchers to take advantage of the benefits of light sheet microscopy.

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