Orthogonal-Plane Fluorescence Optical Sectioning: a technique for 3-D imaging of biomedical specimens

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Techniques allowing to study and visualize intact biomedical objects in high-resolution and in three dimensions have become very popular in the last two decades. Each method, however, suffers from its own specific limitations.

In 1993, a new microscopic and tomographic technique emerged which, because of its many advantages, is growing in popularity and in its applications. The method is called *Orthogonal-Plane Fluorescence Optical Sectioning* and started a new era in microscopy.

This chapter positions the new method among its alternatives and discusses improved versions. We illustrate several applications of the optical sectioning technique on different biomedical specimen types used in our research fields.

Keywords optical sectioning; high-resolution; morphology; tomography; seahorse; gerbil; tadpole; mice

1. Introduction

1.1 Some established tomographic techniques

A popular technique for morphological and structural research is Serial Histological Sectioning. In this method, thin sliced sections of fixed, embedded and stained tissue are created, which are then imaged under an optical microscope. A large amount of histological detail can thus be observed with sub-micrometer resolution for cross sectional images of macroscopic biomedical objects. Obtaining these slices is however extremely work-intensive, requires physical (one-time and one-directional) slicing and thus destruction of the specimen. The method requires semi-automatic to manual aligning and image registration of the recorded slices in order to get realistic 3-D reconstructions. Often image processing of the sections is needed because of the geometrical distortions from the slicing.

To counter the disadvantages of histological sectioning, the development of optical tomographic imaging techniques came about. As these methods create virtual cross sections through a sample using light, it is said that they perform optical sectioning. This approach is non-destructive and perfectly conserves the topographical position of the cross sections in the specimen. As no physical slicing is performed, the sectioning can also be repeated several times over and/or in different orientations.

Several advanced microscopy techniques, such as Confocal Microscopy (CM), allow to obtain high resolution views of certain specimen types. However, because of limited penetration depth the method cannot be applied on large and non-transparent objects. CM uses point-by-point scanning in three dimensions, so a long recording time and inherent bleaching of the specimen are inevitable. Another disadvantage is that light originating from above or below the focal plane will be incorporated in the section image, thus again bleaching but also blurring the section information through out-of-focus interference which decreases longitudinal resolution.

Other wide-spread and powerful alternatives are: X-ray micro Computed Tomography or μCT, which records shadow transmission images of X-ray absorption (~ bone tissue), and Magnetic Resonance Imaging or MRI, which is sensitive for water content (~ soft tissue). The usually attainable resolution with these techniques is about 10 μm for objects of macroscopic scale, and they require extensive calculations to reconstruct virtual cross sections. Recent development in X-ray tomography make it possible to reach higher resolutions (even going below micrometer level) [1], and to visualize soft tissue using phase information or staining.

1.2 A new approach to microscopy

Starting from 1993, another technique has gained much interest and is being developed vigorously: Orthogonal-Plane Fluorescence Optical Sectioning (OPFOS) microscopy or tomography. As this technique delivers 2-D section images with histological information, of soft tissue and bony structures simultaneously ánd in real-time, it is a powerful addition to the established methods. In the OPFOS method, parallel optical sections through a macroscopic biomedical specimen are created by means of a thin laser light plane or sheet, and the omni-directional fluorescence within the sheet is recorded orthogonally. From such a recorded sequential stack of parallel OPFOS recordings, 3-D models can be constructed using dedicated segmentation and mesh generation software.

The OPFOS method is based on two techniques, each about one hundred years old, and was first introduced by Voie et al. (1993) [2].

- In most microscopy techniques, the same optical path and components are used for the illumination and the observation of light. Siedentopf and Zsigmondy (1903) separated the illumination and viewing axis in their Ultramicroscopy technique [3]. This is the first principal technique utilised in OPFOS, however, it was not used for tissue microscopy but rather to study particles in colloidal solutions. By separating the illumination and imaging axis in OPFOS, the sample avoids bleaching in regions that are not being imaged (opposed to CM).
- The other historical procedure reintroduced for OPFOS was initially proposed by Spalteholz (1911) [4]. This method is capable of making all the tissue of the biomedical specimen transparent by matching the refractive index throughout the entire object volume by means of a mixture of oils with refractive indices close to that of natural soft tissue. When the prepared specimen is submerged in this Spalteholz fluid, it appears invisible. This refractive index matching is essential for the OPFOS technique to succeed in creating a penetration depth of tens of millimetres (otherwise it would be limited as in CM).

When required, Voie utilises yet another method in conjunction with the two previous techniques:

- When the specimen contains bony structures or calcified tissue, the calcium first needs to be removed before the Spalteholz procedure is applied. Bone can not be made transparent, as the calcium atoms scatter light strongly. To achieve this decalcification process, a chemical called EDTA is used, which through a chelating process transports calcium atoms outside of the specimen. This is a slow diffusion process which is much accelerated by microwave radiation (without heating) [2,5].

Voie (1993) was the first to combine these methods in the development of a technique that makes virtual cross sections with light sheets through biomedical tissue, like the cochlea in the inner ear [6]. He created laser light sheets according to the Ultramicroscopy technique and, to allow the laser light sheet to be able to pass unscattered and without refraction through the macroscopic object, he applied the decalcification and Spalteholz technique. Only at the intersection of the thin light sheet with the transparent object, omni-directional (auto)fluorescence is excited in the specimen. This light can then be recorded orthogonally to the plane of the sheet. The result is a 2-D virtual optical cross section.

1.3 Overview of the light-sheet fluorescence microscopy field

After the introduction of OPFOS, similar or improved designs and setups were issued under different names. All of them essentially use the same spatial arrangement of illumination and detection like Voie (1993) and Zsigmondy (1903), and all require transparent specimens. In general, this new microscopy branch and family of OPFOS techniques is referred to as (laser) Light-Sheet based Fluorescence Microscopy or LSFM [7]. A short overview of some different implementations is given below.

The first derived design to emerge after Voie et al. (1993) and Voie (2002) was Selective Plane Illumination Microscopy or SPIM by Huisken et al. (2004) [8]. It does not use Spalteholz fluid and is thus capable of imaging small living specimens. These animals, however, need to possess a natural transparency of their own (like Drosophila larvae, Zebrafish and Medaka embryos). This technique is suited to visualize organogenesis in 3-D. Swoger et al. (2007) improved the image quality of SPIM by postprocessing multiple multiview 3-D recorded datasets [9].

A typical drawback of all OPFOS-like techniques are inherent striped line artefacts in the image: when laser light passes through a sample and encounters a particle or region of less(er) transparency, the light behind it is attenuated, creating shadowy dark stripe in the OPFOS image. Huisken et al. (2007) countered this problem with their new multidirectional SPIM or mSPIM setup [10], by adding optical components illuminating the sample consecutively from opposing directions. The resulting two images are computationally fused yielding a superior image.

Other versions of OPFOS implementations, f.i. our newest HR-OPFOS setup [11] or [12], adopted this useful 2-sided illumination approach (however, not *consecutively* but at the same time in one recording). Using the bi-illumination for the laser sheet sectioning, the shadow problem (and the depth attenuation of light) is greatly reduced and clearly improving image quality.

The latest version of SPIM devices has been given a new name: Digital Scanned Laser Light-Sheet Microscope or DSLM [13], as it uses an entirely new configuration to generate the laser sheet. Instead of using a cylindrical lens on a broadened laser light bundle to focus the light in one dimension to a sheet (see more about this in section 2.2), the sheet is now created by a spherical lens and a rapidly moving mirror. In this way, the two-dimensionally focused light is *scanned* into a virtual sheet.

While the SPIM family [8-10,13] is more dedicated to small (microscopic) samples, in which it is easier to obtain good resolution, the original OPFOS technique [2,6] and a derived version renamed to Ultramicroscopy by Dodt et al. (2007) [12] have the capability to image cross sections through macroscopic objects of ten(s) of millimetres in dimension. However, because the sectioning resolution (sheet thickness) is inversely related to the object width that can

be imaged, there is a compromise between the size of the object and the obtainable resolution. To improve the axial slicing resolution on macroscopic objects, we developed the High Resolution OPFOS or HR-OPFOS idea and setup (see section 2.2) [14,15].

A recent publication by Santi et al. (2009) follows the same approach as HR-OPFOS. It builds upon the original OPFOS idea, our modification towards High Resolution OPFOS, and the dual-illumination sectioning from mSPIM. This setup was baptized with yet another name, i.e. Thin-Sheet Laser Imaging Microscope (TSLIM) [16]. As said before, our newest version of the HR-OPFOS setup [10] is also equipped with the 2-sided illumination as in multidirectional SPIM, cf. figure 3.

Recently the MicroImaging department of Carl Zeiss took an interest in the OPFOS technique [17]. A prototype is being developed and will become commercially available in the near future. Their setup is configured to work with small embryos and cell cultures, so not with objects in the macroscopic range of HR-OPFOS, Ultramicroscopy or TSLIM. Zeiss has many dedicated microscopy techniques, each focusing on either flexibility, imaging depth, speed or resolution. The OPFOS family has *all* these benefits according to Zeiss [18] and the active LSFM community [7].

2. The OPFOS technique

2.1 Specimen preparation

Before explaining the OPFOS and HR-OPFOS setups in detail, a short summary of the elaborate but essential specimen preparation is given. Before OPFOS measurements can be performed, the specimen first needs to be bleached*, decalcified*, dehydrated, cleared (refractive index matched), and stained* with a fluorescent dye (* if necessary).

If the specimen contains dark pigmented tissue (f.i. skin or eyes) it is first bleached with a 5% hydrogen peroxide solution. Specimens containing bone or (mineralized) cartilage are decalcified in a water solution of 10% EDTA [2]. This process is much accelerated by exposing the specimen to low power microwave radiation without heating [5]. After all calcium has been removed, which might take several days using microwave acceleration (compared to weeks or months without microwave radiation), the sample is fully dehydrated in a graded series of ethanol concentrations. This dehydration step is required to apply the Spaltholz procedure. The Spalteholz fluid [4] is a mixture of oils (three parts of benzyl benzoate and five parts of methyl salicylate) which is insoluble in water but soluble in ethanol. Remaining water in the specimen therefore adversely affects the image quality of the sections. The actual clearing is achieved by putting the object in gradually climbing concentrations to 100% Spalteholz fluid in ethanol, each step taking 24 hours. In the end, the refractive index of the tissue is practically identical to the surrounding (Spalteholz) fluid, so that neither scattering nor refraction of light occurs: the specimen is completely invisible when submerged in the fluid. Then, the object can be made (extra) fluorescent by putting it in Spalteholz fluid containing a low concentration of fluorescent dye, for instance 5.10^{-4} mg/ml of rhodamine B (absorption-emission 543-570 nm) with a green laser.

The decalcification process can not be undone. However, the preparation steps that follow are partially reversible. The specimen can be taken through a backward graded Spalteholz fluid and alcohol series back to ‘water’, but artefacts can not be excluded. So in practice, the whole refractive index matching procedure should be regarded as non-reversible, which can be a drawback if one wants to use or compare the specimen data with other methods afterwards.

2.2 (HR-)OPFOS setup

Figure 1 shows the schematic layout of our OPFOS setup. The object is illuminated by an XY-sheet of laser light travelling along the X-axis, and the fluorescence light emitted in the direction of the positive Z-axis is used for imaging. Images of virtual slices in the XY-plane will thus be recorded, and by translating the object along the Z-axis, a sequence of section images is obtained.

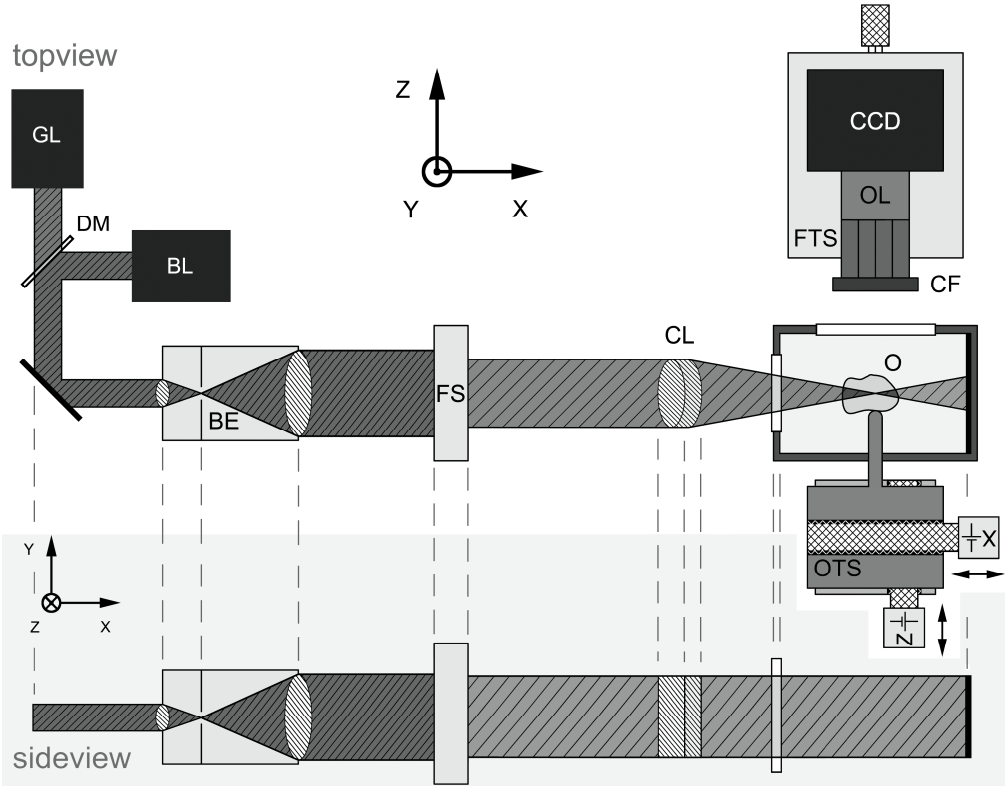

Fig. 1 Schematic drawing of the (HR-)OPFOS setup: Light from a green (GL) or blue laser (BL) passes through a Keplerian beam expander (BE) with spatial filter, a field stop (FS) and a cylindrical achromat lens (CL) which focuses the laser along one dimension within the transparent and fluorescent object (O). A two-axis motorized object translation stage (OTS) allows scanning of the specimen and imaging of different depths. The fluorescence light emitted by the object, is projected onto a CCD-camera by a microscope objective lens (OL) with fluorescence colour filter (CF) in front. The focusing translation stage (FTS) is used to make the objective lens focal plane coincide with the laser focus.

An essential part of the OPFOS setup is the generation of a sheet of laser light. In reality, it is impossible to generate a perfect plane or sheet of light. In practice, a cylindrical lens focuses a laser beam along one dimension to a beam with a hyperbolic light profile. This hyperbola is shown in figure 2. The dark gray area in the centre represents the *1/e²* intensity profile. The thickness of the profile increases along the X-axis as one moves away from the focus d_1. Within the so called Rayleigh range x_R, the hyperbolic intensity area can be approximated by a rectangle: within this rectangle an object is sectioned by a light plane of approximately constant thickness. The Rayleigh range x_R is the distance on either site of the minimal focus d to where the hyperbolically focused beam has thickened to $\sqrt{2}\,d$ and where the approximation as a plane is valid. It is given by the expression $b = 2x_R = \frac{\pi d^2}{2\lambda}$, where b is called the confocal parameter, or the total distance in which a focus smaller than $\sqrt{2}\,d$ is maintained. One can notice that a larger focal thickness d goes along with a larger confocal parameter b.

- In conventional OPFOS, a 2-D image is taken over the full width $\Delta x = b_1$ of the XY-sheet. The object under study consequently has to fit within this zone. So, a trade-off exists between maximal image width ($\approx b_1$) and the sectioning thickness $\sqrt{2}\,d_1$ ($\sim 1/\sqrt{b_1}$). In summary, an OPFOS image has a slicing thickness d_1 in the centre, growing to $\sqrt{2}\,d_1$ at the edges x_R. Everything within the thickness of the laser light sheet is integrated into a flat picture (so actually a varying thickness and slicing resolution is present in the 2-D image).

We introduced a simple but useful modification to the OPFOS setup, which delivers a much higher axial resolution:

- In High Resolution OPFOS, we no longer record a 2-D image along the entire object width, but we scan the object through the line of best focus, created by a large aperture cylindrical lens. Using such a large aperture lens, a much smaller focus d_2 is obtained, and consequently the confocal parameter b_2 becomes very small, cf. figure 2. Too small in fact to fit an entire (macroscopic) object inside. This is, however, not a problem because we translate the object step-by-step along the X-axis and only record the consecutive image column within the zone/line of best focus. So, in HR-OPFOS the sectioning thickness is no longer a compromise with image

width, and is in theory only limited by diffraction. Furthermore, the sectioning thickness is constant and equal to d_2 in the entire 2-D image. A logical disadvantage of this HR-OPFOS approach, however, is that the 2-D cross sections are no longer created in real-time, as in OPFOS, but rather stitched from line-scanning.

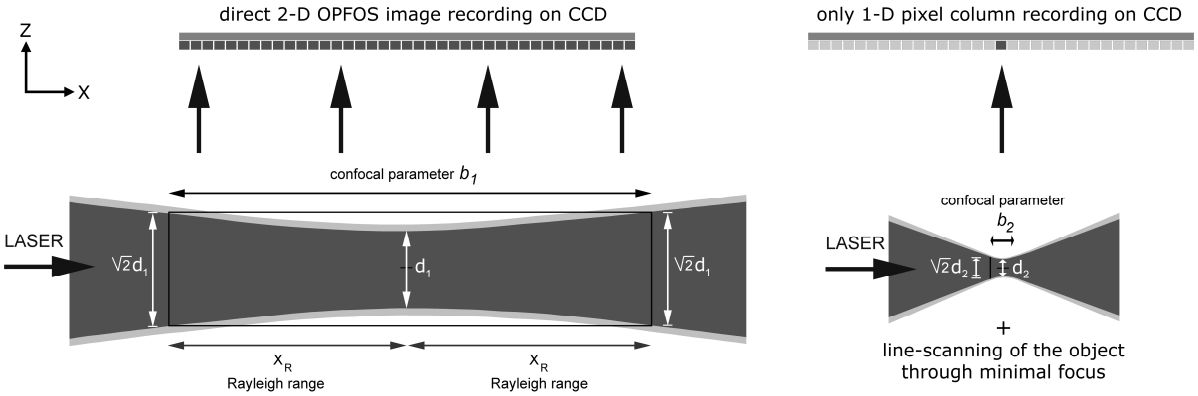

Fig. 2 *left*) Hyperbolic focus profile of a small aperture cylindrical lens. A long confocal parameter b_1 is obtained in which the focal thickness d_1 remains *approximately* constant. In OPFOS, 2-D images are recorded of this approximated planar sheet in the confocal zone.
right) Focusing profile of a large aperture cylindrical lens. By making the focal thickness d_2 very small, the confocal parameter b_2 consequently becomes much smaller as well. HR-OPFOS images are obtained by recording only 1-D pixel lines in the minimal focal zone and by scanning the object through the focus from left to right along the X-axis.

The lateral resolution of the (HR-)OPFOS methods depends on the camera spatial resolution and the numerical aperture of the (magnification) lens used. The axial resolution depends on the thickness of the laser light sheet, as all the information within the thickness of the sheet is integrated to a flat picture. In HR-OPFOS, we achieve a slicing axial resolution up to 2 µm. The in-plane resolution is up to 1 µm. Specimens can also be stained with fluorescent dyes, like rhodamine B, for extra contrast.

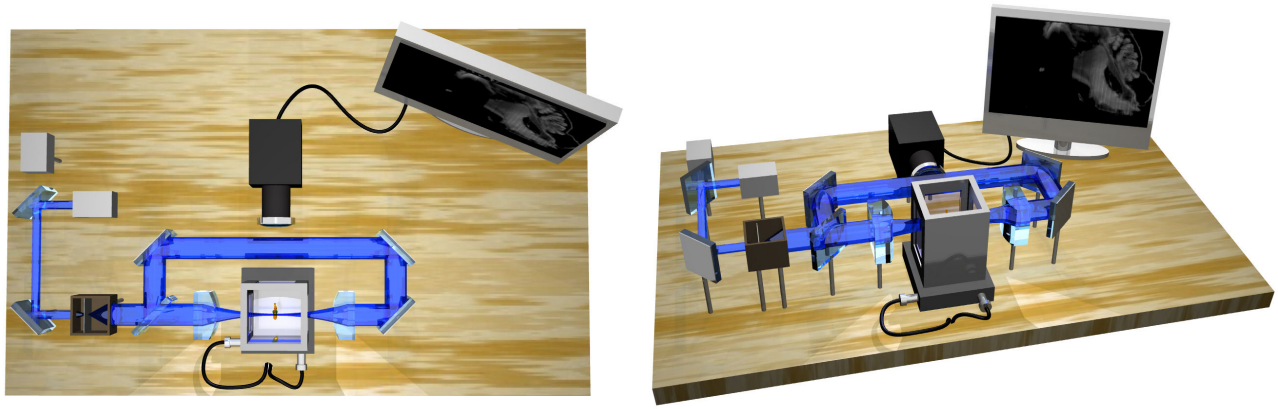

Fig. 3 An *artist* impression of our HR-OPFOS setup with two-sided cylindrical lens sheet illumination, and with two laser wavelengths (green and blue). The green laser (532 nm) is suited to excite rhodamine B fluorescence, while the blue laser (488 nm) is suited for excitation of autofluorescence in many biomedical tissue samples.

Some advantages of OPFOS can now be summarized.

- The technique is optical and non-destructive in nature.
- It achieves high-resolution both in-plane and longitudinal, showing histological detail.
- Out-of-focus fluorescence is not present, as fluorescence can only originate from within the laser sheet (identical to the focal plane of the recording device).
- Only the observed plane through the specimen is illuminated and therefore affected by bleaching.
- Functional staining is compatible with the technique.

- Image registration between subsequent slices is achieved automatically.
- Multiple recordings, f.i. of different slicing directions, are possible.
- The method is fast and capable of performing real-time virtual sectioning.
- The method is suitable to image bone and soft tissue structures simultaneously.

Inevitably, also some disadvantages exist:

- The specimen preparation is elaborate, prone to artefacts and mostly irreversible.
- Large amounts of data are recorded.
- Because of the typical stripe or shadow artefacts, it can be necessary to perform manual image segmentation.

3. Application examples

3.1 Middle and inner ear research

The first demonstration and application of the OPFOS method is situated in the field of hearing research, where we study the morphology and mechanics of the hearing organ. Dissected ears of the laboratory animal gerbil (*Meriones unguiculatus*) are used and prepared to be compatible with OPFOS as described in section 2.1. HR-OPFOS scanning delivers the optical cross sections, shown in figure 4 and 5. Through segmentation and mesh generating software (e.g. Amira (Visage Imaging)) we can make the 3-D model shown in figure 6, clearly containing soft tissue (muscle, tendon and blood vessel) and bone.

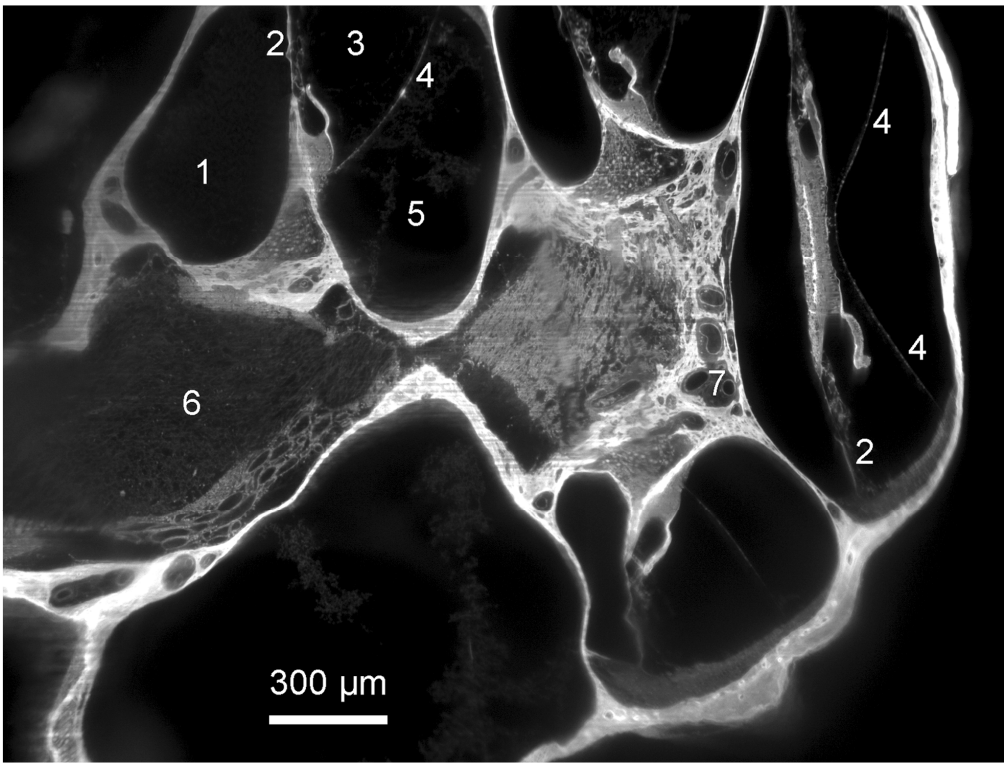

Fig. 4 A cross section of a cochlea in a right Gerbil inner ear of 1.92 x 2.55 mm (1280 x 1700 pixels). HR-OPFOS achieved a lateral and axial resolution of 2 µm on rhodamine B stained tissue.
1. scala tympani, 2. basilar membrane, 3. scala media, 4. Reissner's membrane, 5. scala vestibuli, 6. modiolus, 7. blood vessels.

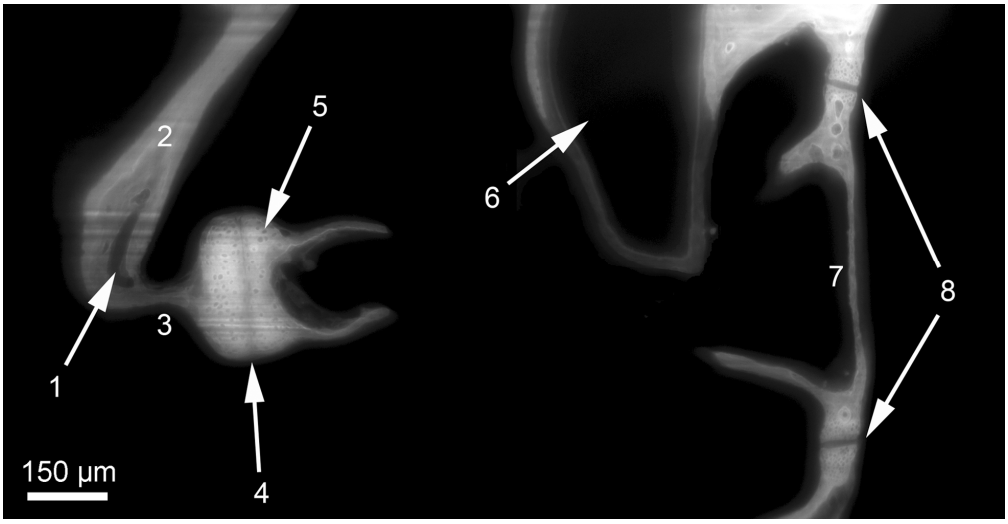

Fig. 5 A cross section of a right Gerbil middle ear of 0.96 x 1.88 mm (1280 x 2500 pixels).
HR-OPFOS obtains a lateral resolution of 1 µm and axial resolution of 2 µm on rhodamine B stained tissue.
1. channel within the anvil, 2. anvil hearing bone, 3. lenticular process, 4. articulation joint, 5. calcified cartilage within head of the stirrup hearing bone, 6. stirrup artery, 7. stirrup footplate, 8. annular ligament footplate of stirrup.

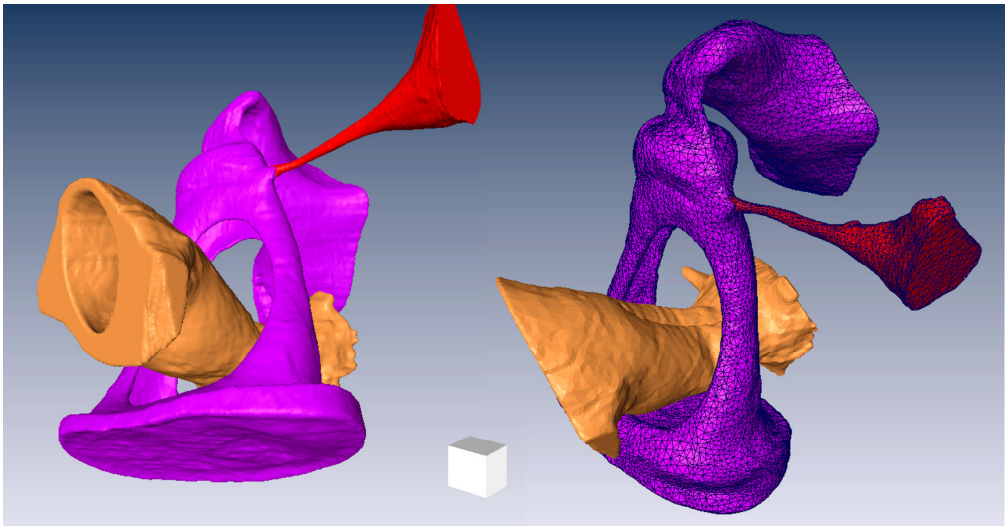

Fig. 6 From a 3-D HR-OPFOS data stack we can create three-dimensional reconstructions. This model showing a part of the middle ear of gerbil originates from a similar data stack as the image in figure 5:
the anvil hearing bone attaches to the stirrup hearing bone, a big artery is running through the stirrup legs and the stirrup muscle connects at the stirrup head (voxel size 1.5 x 2 x 2 µm). The dimensions of the white cube are all 150 µm.

3.2 Mice brain research

In the field of neurology, imaging of the brain is used to gain insight into its morphology and to create brain atlases. OPFOS microscopy of brain and nerve tissue is, however, difficult as myelin sheaths surrounding the nerve axons are immune to the refractive index matching process of the Spalteholz method. Another problem is that Spalteholz fluid deteriorates or entirely demolishes GFP(-labelled) fluorescence, which is often used in brain research. Nevertheless it is possible to apply OPFOS on young mice (C57 black *Mus musculus*) or birds for instance, which do not yet have much myelin in the brain. Some cross sections of a mice brain are shown in figure 7. The OPFOS image data allows to distinguish between different brain tissue structures and regions.

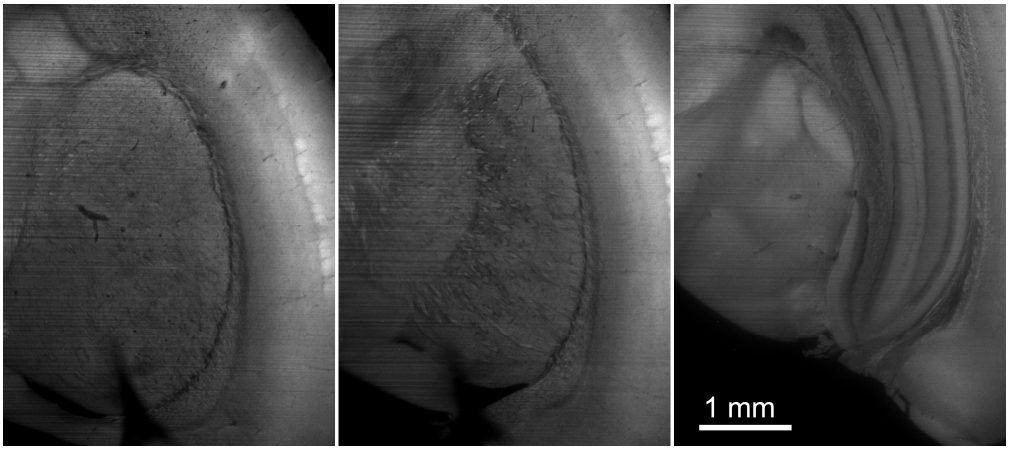

Fig. 7 Three OPFOS cross sections (4.8 x 3.6 mm) at different depths through a mouse brain. OPFOS recorded the natural autofluorescence of the specimen with a blue laser.

3.3 Seahorse musculature research

When studying the morphology of vertebrates, the shape, function, working principles and attachment method of muscles are important topics. Series of 2-D HR-OPFOS sections through young long-snouted seahorses (*Hippocampus reidi*) were used to study their musculature used in feeding. Using OPFOS we imaged the head and focused on the sternohyoid muscle, a thin narrow muscle attaching the hyoid bone to the pectoral girdle. It allows the seahorse to increase the volume of the buccal cavity very rapidly, and so suck in its prey with great force [18]. Because this muscle is extremely small in newly born seahorses and a 3-D interpretation of its functioning and volume was desired, OPFOS was applied with clear and interesting results, cf. figure 8.

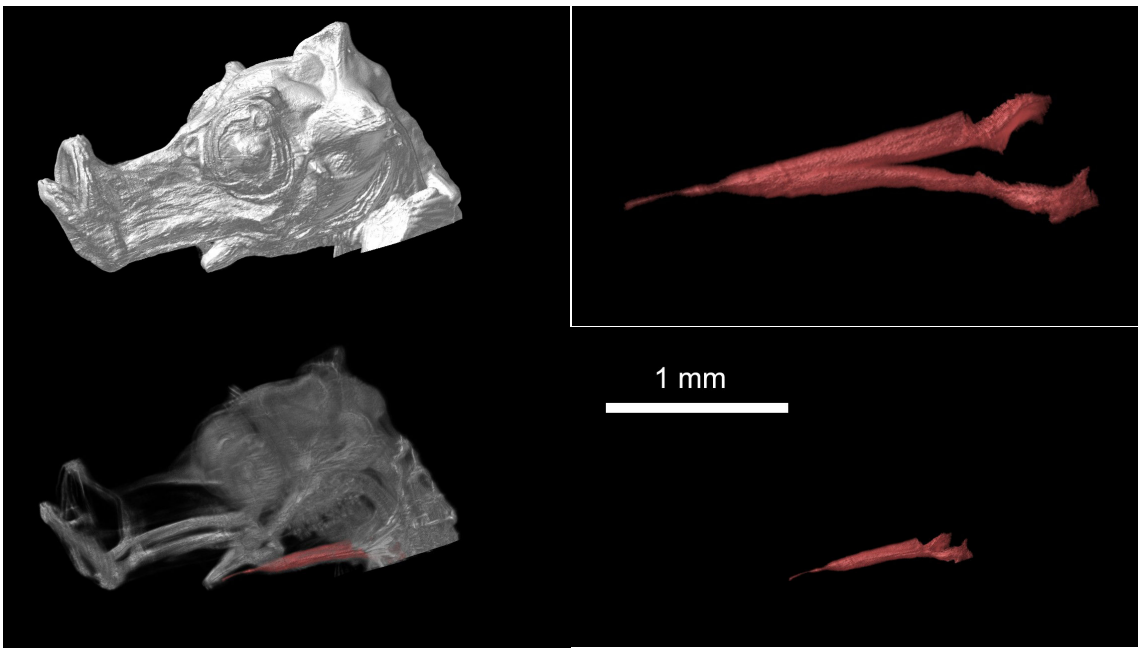

Fig. 8 Functionally segmented 3-D model of the sternohyoideus muscle from OPFOS data from a one-day old *Hippocampus reidi* seahorse. It clearly reveals small morphological detail in the muscle shape and in its relation to the bones to which it is attached (voxel size 3.5 x 3.5 x 5 µm).

3.4 Frog tadpole organogenesis research

Another subject when studying development and evolutionary morphology of small vertebrates lies in organogenesis. The objects under study are now young African clawed frog tadpoles (*Xenopus laevis*). Up till now, mostly serial histological sectioning was used to study the morphology and development of tadpole organs and structures, and to build 3-D models from. A comparison of the obtained results from histology and OPFOS showed many benefits for the

latter [19]. Apparently, geometrical distortions, image registration and especially shrinking artefacts related to the specimen preparation in histological sectioning ruin the three-dimensional topography and proportions of the images, and consequently of the reconstructed models. The OPFOS 3-D reconstruction in figure 10 is clearly very detailed and without big proportional distortions [19].

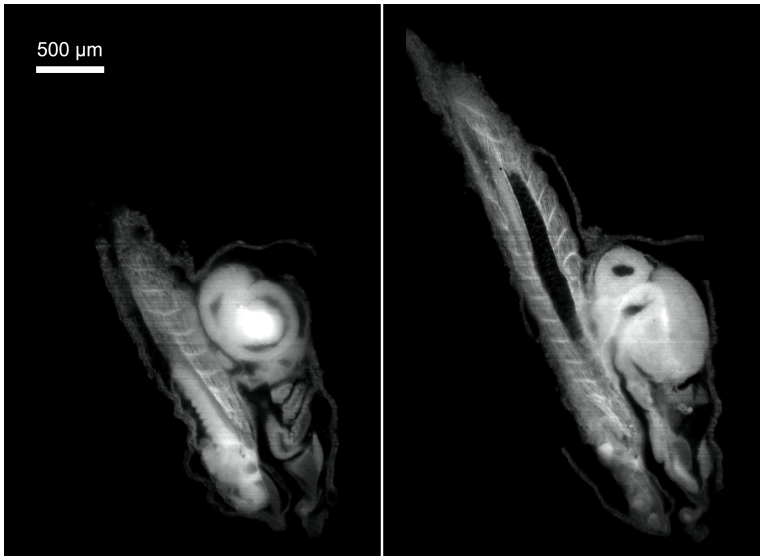

Fig. 9 Two cross sections of 1200 x 1600 pixels (2.7 x 3.7 mm) at different depths through a frog tadpole body. The image shows the segmented body musculature, the brain and digestive tract. OPFOS recorded the natural autofluorescence of the specimen with a blue laser.

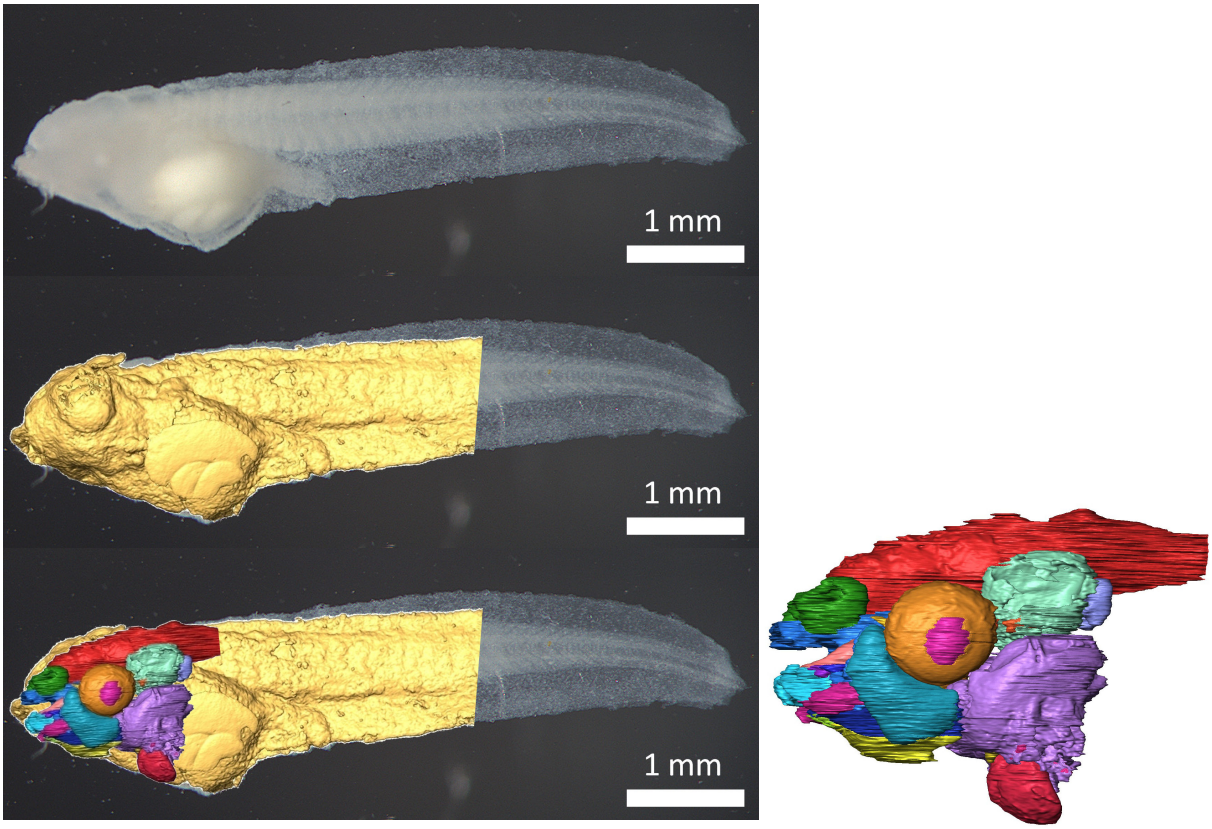

Fig. 10 Photograph superposed with external OPFOS surface model and internal OPFOS organ models. The models shown here originate from the same data stack as the images in figure 9. Different colours indicate different organs comprising sensory organs, muscles, cartilaginous structures and neuronal structures (voxel size 1.5 x 1.5 x 3 µm).

4. Conclusion

A relatively new high resolution optical method, called Orthogonal-Plane Fluorescence Optical Sectioning microscopy or tomography, has been introduced. This OPFOS method allows to generate images of virtual cross sections through different kinds of biological specimens. From these sections, full and detailed 3-D shape data is obtained. Despite its conceptual simplicity, (HR-)OPFOS generates highly detailed section images of bone as well as soft tissue, in nearly real-time and under a variety of orientations.

In this chapter, we gave an introductory overview of the OPFOS history, its working principles and state-of-the-art. Furthermore, we illustrated the applicability of the method on several specimen types from different research areas, proving its flexibility and possibilities. The technique has lots of (even undiscovered) potential and fills a void between other tomographic microscopy techniques.

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