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UltraMicroscope: Applications & Literature
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Minireview COMMERCIAL

The UltraMicroscope is the most successful commercial light sheet system for high impact publications

LaVision BioTec, UltraMicroscope Division[1]

The UltraMicroscope serves diverse applications. These share the fact that imaging only a small part of the sample is not sufficient for analysis and distorting artifacts introduced by sectioning are sought to be avoided. Researchers who need artifact free data from overview to a specific region of interest implement this technology into their projects. Here we give a brief overview regarding applications from *in vivo* imaging to clearing and list the current UltraMicroscope articles.

[1]LaVision BioTec GmbH, UltraMicroscope Division, Astastr. 14, 33617 Bielefeld, Germany, Tel: +49 (0) 521 915139-0, Fax: +49 (0) 521 915139-10, http://www.lavisionbiotec.com
Correspondence: schroeer@lavisionbiotec.com

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Abbreviations: UM, UltraMicroscope; BABB, Benzyl Alcohol-Benzyl Benzoate; CLARITY, Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-compatible Tissue-hYdrogel; CUBIC, Clear, Unobstructed Brain/Body Imaging Cocktails and Computational analysis; DBE, Dibenzyl Ether; DCM, Dichloromethane; iDISCO, Immunolabeling-enabled 3D Imaging of Solvent-Cleared Organs; LSM, Light Sheet Microscopy; PACT, Passive CLARITY Technique; PARS, Perfusion-assisted Agent Release in Situ; PFA, Paraformaldehyde; RI, Refractive Index; RIMS, Refractive Index Matching Solution; SDS, Sodium Dodecyl Sulfate; SWITCH, System-Wide control of Interaction Time and kinetics of CHemicals; THF, Tetrahydrofuran; uDISCO, ultimate 3D Imaging of Solvent-Cleared Organs; 3DISCO, 3D Imaging of Solvent-Cleared Organs

INTRODUCTION

The history of the light sheet microscope began in 1903 with its invention by Siedentopf and Zsigmondy (1). The ultramicroscope described here was used for the visualization of particles below the diffraction limit in a solution by means of a non-coherent light sheet. It was a long time before there were any further significant developments in this field. The concept of orthogonal, planar illumination was used mainly in the field of flow cytometry (2). It took until 1993 for the concept of the light sheet microscope to be taken up again by Voie (3). He made use of the light sheet technology known as orthogonal-plane fluorescence optical sectioning (OPFOS) for the characterization of cochlea. In 2004, the OPFOS concept was again reported in an article by Stelzer (4).In this, researchers used a light sheet microscope for the visualization of live samples. In particular, the low phototoxicity of this technology has been used to observe in vivo Medaka embryos and Drosophila melanogaster larvae over several hours. The advantages of light sheet technology in terms of the characterization of large clarified samples were presented in work by Dodt, 2007 (5). This time the use of light sheet technology was combined with sample-clearing, as described by Spalteholz in 1914 (6). This very powerful combination made it possible to generate 3-dimensional representations of intact specimens. It also became possible to exclude incorrect results caused by the cutting of the sample. Also researchers were no longer restricted to minor biopsies. At that time, there was no commercial technology for the implementation of this method of analysis. Then, in 2009, LaVision BioTec presented the first commercial light sheet microscope since the one created by Siedentopf and Zsigmondy. The LaVision BioTec UltraMicroscope was developed for the study of large, clarified samples. The provision of this technology formed the basis of a global development movement in the area of sample preparation. It made it possible for different clearing procedure to be developed and optimized. Outstanding clearing procedures such as the CLARITY (7), CUBIC (8), 3DISCO (9) and the iDISCO (10) protocols were developed by research groups working with the UltraMicroscope. In 2012, the Federal Ministry of Economics and Technology awarded LaVision BioTec the 2012 ZIM Prize in recognition of the paramount importance of Ultramicroscopy in research and innovation and their boost to this proven system.

ABOUT SAMPLE CLEARING

Imaging large samples needs certain procedures to reduce the opacity. Some organisms like Zebra Fish larvae are mostly transparent by nature, but the majority of samples are opaque making attempts to image a sample in depth difficult. Considering *ex vivo* samples, there are two main principles of creating translucent samples that have been established to this day. In the case of organic solvent clearing, the principle of operation is equalizing the refractive index of sample and solution. On the other hand, the sample may be cleared by using aqueous buffers which have a certain depolymerizing effect on structures like lipid chains.

Organic Solvent Clearing Protocols
When performing organic solvent clearing, the water has to be removed in the first step by incubating the sample in increasing concentrations of methanol or another dehydrating solution. After this step, the refractive index of water (1.33) is virtually no longer present. Within a second step, the remaining refractive indices are matched by an organic solvent. The organic solvent clearing leads to very transparent samples and is perfectly suited for dense tissue like tumors, adult tissue or highly myelinated brain. The majority of immuno-histochemical staining is well conserved. To preserve the fluorescence of proteins like GFP, certain protocols like FluoClearBABB or uDISCO have to be applied. The UltraMicroscope II can be used for all current organic solvent clearing procedures including BABB and iDISCO. Currently following organic solvent based protocols have been published: BABB (6), FluoClearBABB (15), THF/DBE (14), 3DISCO (9), iDISCO (10, 16), iDISCO + (25), uDISCO (24) and ethyl cinnamate (26).

Water-Based Clearing Protocols
The most common operating principle of water-based clearing protocols is by depolymerization. By dividing large structures like lipid chains into small micelles of different sizes, the opacity is remarkably reduced. As a depolymerizing reagent, aqueous buffers can contain urea as it is used for CUBIC clearing. A SDS buffer and an advanced electrophoresis protocol are used for CLARITY clearing. The clearing protocols differ in complexity and in the degree of translucency which can be achieved. By depolymerization, the entire structure of a sample can be debilitated while the fluorescence of proteins like GFP is well preserved. These aqueous buffer based clearing protocols have been published: Scale, ScaleA2, ScaleU2 (20), SeeDB, SeeDB2 (21), FRUIT (18), CUBIC (8, 11, 12), ClearT, ClearT2 (17), CLARITY, PACT-PARS, CLARITY2 (7, 19), SWITCH (22).
Both organic solvent-based and water-based clearing methods are powerful tools for successful sample preparation. The variety of clearing protocols shows that clearing procedures have to be optimized for the sample of interest. The UltraMicroscope II is capable to handle all current clearing solutions.

DISCUSSION

What kind of clearing is best for my project?
There are several protocols for tissue clearing available today and it is not really easy to identify the best protocol for your sample. There are two large groups of clearing procedures: the aqueous buffer based protocols and the organic solvent clearing. Every procedure has its advantages and disadvantages. If you start with aqueous buffer based clearing please consider that samples might swell and get very soft because the stabilizing structures will be depolymerized. Furthermore, it might take quite a while to get the samples cleared. Another disadvantage might be a stronger background and some turbid appearance of the tissue. But this is not the case for all aqueous buffers based clearing protocols. From what users report we know that CLARITY (7) and CUBIC (8, 11, 12) deliver good results. The CUBIC protocol is not very complex and can be followed in detail from the different publications. A brain hemisphere is ready for imaging within about 11-13 days. Chemicals which are needed for this kind of clearing are not that expensive and the CUBIC buffer R2 is very useful for imaging samples cleared with RapiClear®. Please consider that there are different RapiClear® (SunJin Lab Co.) solutions available with different refractive indices. The CUBIC samples do not swell that much and they get really clear. The CLARITY protocol also generates perfect samples but it is a bit more complex. It may take a while to establish this protocol in your lab but today there are also systems available like the EasyClear® (LifeCanvas Technologies) doing the clearing for you. For the imaging solution of CLARITY samples one can choose between different liquids like FocusClear® (CelExplorer Labs Co.), 80% glycerol, 63% thiodiethanol or RIMS (refractive index matching solution) (13). You may also try the CUBIC R2 buffer. There might be a slight difference regarding the refractive index between the sample and the imaging solution. In that case just let the solution settle for 20 minutes and then start imaging. For further assistance on how to use CLARITY you can visit http://clarityresourcecenter.org/

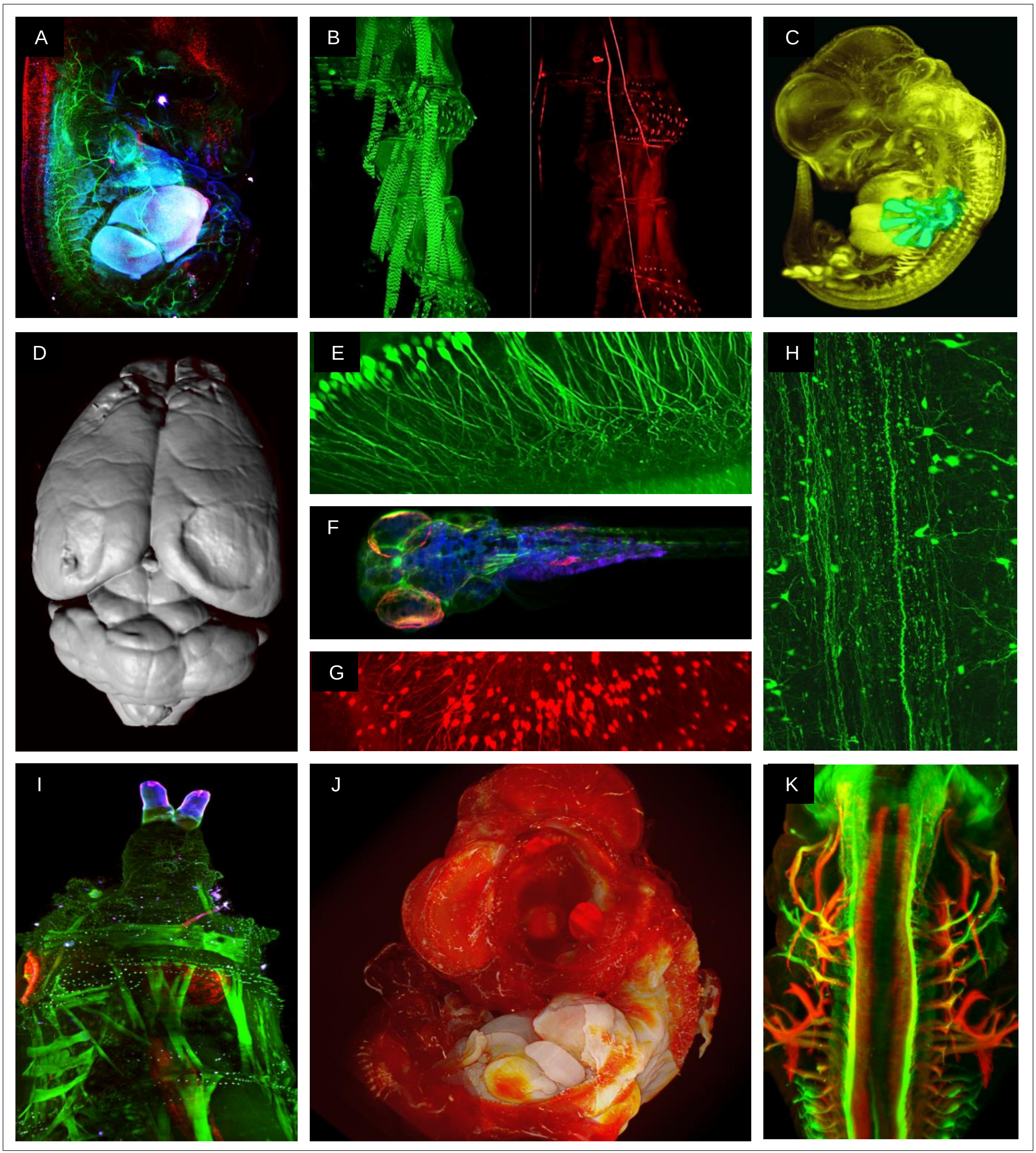

Fig. 1: UltraMicroscope applications – A) mouse embryo triple staining; B) *Drosophila melanogaster* larvae; C) mouse embryo; D) Brain lesion; E) mouse hippocampus, GFP; F) Zebra Fish *in vivo*; G) mouse frontal cortex; H) mouse spinal cord, GFP; I) *Drosophila melanogaster* larvae, GFP & autofluorescence; J) chicken embryo, autofluorescence; K) mouse embryo, iDISCO;

There are several further aqueous buffers based clearing protocols but some of them are only suitable for embryonic tissue. Other procedures require a buffer which is very viscous and is not really easy to handle due to the formation of small bubbles inside. Some protocols are very time consuming with incubation times of several weeks. There are also protocols generating tissue which is like gel and expanded to more than twice the original size.

Very often it is difficult to clear adult and dense tissue only with aqueous buffers. In that case you may try the other group of clearing protocols which is based on organic solvents. The first who described this technique was Werner Spalteholz (6). After using clove oil he searched for solvents to clear tissue. Besides the methyl salicylate protocol a mixture of benzyl alcohol and benzyl benzoate showed convincing results. This protocol is still in use today. Users should always be aware of the fact that this clearing solution is very harmful and should only be handled under a fume hood. A less harmful clearing procedure is based on tetrahydrofuran and benzyl ether (14). This protocol is today mostly known as the 3DISCO protocol (9). The advantage of organic solvent clearing is that samples are getting as clear as glass. The clearing itself is fast. Depending on the size of the sample it can be done within a few hours. For a long time, the major disadvantage of these protocols was that the fluorescence of GFP and other fluorescent proteins was not preserved. Nowadays, this issue can be circumvented. On the one hand, the FluoClearBABB (15) and uDISCO (24) preserves GFP very well while clearing the sample. On the other hand, GFP can be immunohistochemically stained following the iDISCO protocol (10, 16). This outstanding protocol describes how to combine the gold standard immunostaining with sample clearing. The advantage is that one can target several proteins without generating the GFP mouse model for each target. This protocol also enables the usage of chromophores in the far red. GFP is excited with 488 nm but this wavelength is not really suitable for deep imaging because light at this wavelength is absorbed and scattered by the tissue resulting in high background and weak target intensities. Immunolabeling and imaging with a far red dye is much more suitable for deep imaging, reducing autofluorescence while increasing imaging depth. Of course, each protocol has to be optimized for your tissue, and antibody; and please be aware that this may take a few attempts until you have the perfect sample. However, there is also great assistance. The iDISCO developers put their knowledge to the internet: http://idisco.info/

Questions and answers

Can I use DAPI? - DAPI is excited with 405 nm. This is a pretty short wavelength which is scattered and absorbed by the tissue. It is absolutely not suitable for deep imaging. One should try DRAQ5™ or TO-PRO®.

My sample contains GFP, which clearing should I try? - You may try uDISCO, ethyl cinnamate, CUBIC, CLARITY, FluoClearBABB or iDISCO. iDISCO maybe of advantage due to the fact that you can label it with a far red dye for a better penetration of the excitation wavelength.

The tissue I am working with is very dense. What clearing is capable to get it transparent? - Users working with dense samples often use BABB or 3DISCO.

The sample I would like to image is colorized (e.g.: liver). How can I cope with colorization? - You may try the iDISCO+ or CUBIC-perfusion protocol. These protocols were designed to decolorize sample tissue by perfusion.

I would like to label my sample with fluorophores which are suitable? - Every fluorophore with an excitation maximum between 500 nm and 785 nm is suitable. The extended wavelength can penetrate the tissue better. DiI or DiAsp are not compatible with most clearing protocols. The membranes as binding site for these dyes is often altered or affected by the clearing. Users have for example good results with using several Alexa Fluor® dyes (Thermo Fisher Scientific Inc.), ATTO-fluorescent dyes (ATTO-TEC GmbH), VivoTag® (PerkinElmer Inc.), Cy7, or IRDye ®(LI-COR, Inc.).

My sample is very soft. How can I mount it for the image acquisition? - You may try some glue. Loctite Professional® or picodent® are even stable in organic solvents.

The sample I would like to image is very small. How can I mount it? - If you work with organic solvents you may prepare an empty but cleared cube with 1% low melting agarose. After clearing the cube you make a small cut on top of it where you insert your cleared and stained sample. One can also directly embed the sample into agarose and do the clearing of the sample within the agarose. In that case please extend the dehydration (100% over night) so that the agarose is completely dry. If there is only a small amount of water remaining in the cube it will start shrinking and it will get milky as soon as it is transferred to the clearing solution. If you work with aqueous buffers you may insert the sample into a FEP tube or a cube of Phytagel™ (Sigma-Aldrich Co. LLC.).

I cleared my sample but it is still not transparent and shows a strong background. -If you have applied an aqueous buffer based protocol you may give a try to an organic solvent protocol. Methanol treatment might reduce the background. Please consider to dehydrate the sample completely. If the used dehydration solution (tetrahydrofuran, ethanol or methanol) is stored in a bottle which is opened frequently there will be already a remarkable amount of water in that solution due to the hygroscopic characteristic of the solvent.

I cleared my sample using the CLARITY protocol. What kind of solution should I use for imaging? - Some CLARITY imaging solutions are very expensive. To reduce costs you may try sRIMS, 63% thiodieethanol or 80% glycerol. *The iDISCO protocol resulted in a sample with a strong labelling only on the* *surface. What should I do?* - Increase the dilution of the antibody and find further information on: http://idisco.info/

MATERIAL AND METHODS

A brief organic solvent clearing protocol

Due to the diversity of current clearing protocols we pick one simple organic solvent based protocol to start with. This protocol will deliver reasonable results within shortest time. A dehydration and a clearing solution are required. For dehydration methanol (MetOH) or tetrahydrofuran can be used. (THF, Sigma-Aldrich). As clearing solution for matching the refractive indices dibenzyl ether is applied (DBE, Sigma-Aldrich).
For small tissues, the 70% (vol/vol) THF or MetOH step can be skipped to save time. Changing the solutions two to three times during the incubation in 100% THF or MetOH and later in DBE will improve the clearing. In case of spinal cord or brain clearing, refresh DBE every 10 min. To optimize the clearing protocol for different tissues not listed here, follow the protocol for those samples which are similar in size and composition as displayed in Tab. 1. If the sample is still not perfectly cleared insight extend the 100% dehydration step by doubling the incubation time and test that there is no water in the dehydration solution.
During incubation prevent exposure to light. Imaging should be done as soon as possible after clearing. The fluorescence will decrease in intensity after a while.

Immunohistochemistry

Immunohistochemical staining has to be done before the clearing procedure starts. The staining with labeled antibodies needs extended incubation times if a whole mount has to be stained. A mouse embryo E14 needs around 5 days per antibody

Samples containing GFP or a fluorescent protein

Extended dehydration will decrease the fluorescence of proteins most widely. For this reason incubate fluorescent protein containing samples as short as possible in dehydration solution. Small cleared samples can be placed later in the cleared agarose cube by cutting a small slit into the cube and inserting the cleared sample. In general the uDISCO, ethyl cinsamate or the FluoClearBABB protocol would be more suitable for GFP samples.

Clearing agarose cubes

Boil 1% low melting agarose (conventional agarose may also work) in A. dest. Pour the agarose solution into a Petri dish (approx. 0.5 -0.8 cm). Wait until it is cooled down for cutting out small cubes (approx.1x1 cm edge length). Transfer a maximum of 5 cubes into 20 ml of 50% THF for 2.5 h at RT followed by 70% for 2.5 h at RT. Final dehydration is done by incubating in 100% for 2.5 h at RT and 100% over night at RT. The first clearing step follows with 100% DBE for 2.5 h at RT and the second step with 100% DBE. Store the cubes at 4 °C until usage. Instead of THF MetOH can also be used.
If the cubes start to shrink and if they get milky whitish as soon as you transfer them into the DBE the dehydration with THF or MetOH was not completed. In that case incubate new agarose cubes for a longer time in a larger volume of 100% THF or MetOH.

Reagents	Mammary gland, lymph node	Spinal cord, lung, spleen, mouse embryo	Brain stem	Brain
50% (vol/vol) THF	20 min	30 min	1 h	1 h
70% (vol/vol) THF	20 min	30 min	1 h	1 h
80% (vol/vol) THF	20 min	30 min	1 h	1 h
100% (vol/vol) THF	20 min, 20 min, 20 min	30 min, 30 min, 30 min	1 h, 1 h	1 h, overnight, 1h
DBE	≥15 min	≥15 min	≥30 min	≥3 h

Tab. 1: Incubation times for 3DISCO clearing (Nature protocols. 2012 Oct, Three-dimensional imaging of solvent-cleared organs using 3DISCO, Ertürk et. al.)

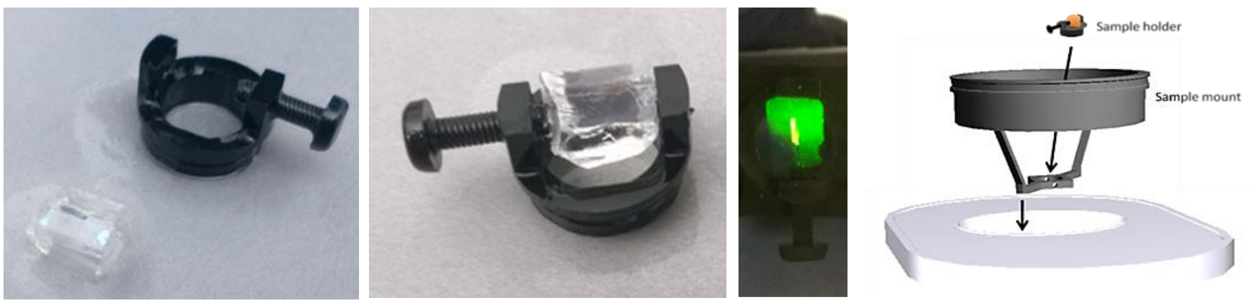

Fig. 2: Sample mounting using cleared agarose cubes

clearing protocol / imaging solution	refractive index
Scale	1.38
SWITCH	1.44
CLARITY	1.45 (1.48)
Clear^T	1.45
FRUIT	1.48
RIMS	1.48
CUBIC	1.49
SeeDB	1.49
SeeDB2	1.52
BABB	1.55
iDISCO /DBE	1.56

Tab. 2: Refractive indices of clearing and imaging solutions.

in vivo imaging
The *in vivo* setup for the UltraMicroscope II ensures constant environmental conditions within the sample cuvette considering the temperature and the CO_2/O_2 atmosphere. All settings are controlled by a touch-screen. The sample feedback mode measures the temperature at the sample with an additional temperature sensor. Thus, the temperature of the thermostat is automatically adjusted to the feedback of this sensor. The heating element and the sample holder can be easily unmounted for autoclaving.

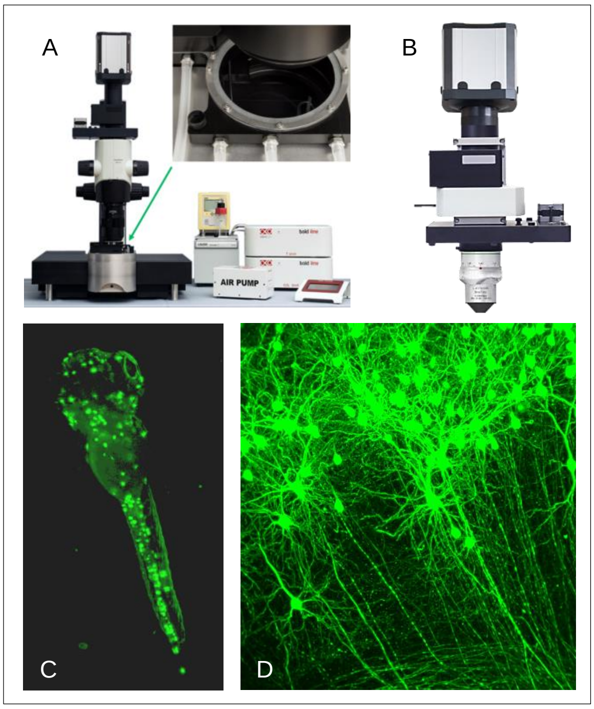

Fig. 3: A) *in vivo* setup for time lapse imaging with environmental control; B) Infinity corrected optics setup; C) Zebra Fish in vivo; D) Mouse hippocampus maximum intensity projection 20x, NA 0.95, BABB objective lens;
High resolution imaging
The UltraMicroscope II infinity corrected optics setup delivers superior imaging capabilities and user friendliness. It can be mounted directly to the focusing unit instead of the zoom body. It allows the implementation of infinity corrected objective lenses like the LaVision BioTec multi immersion objective lenses.

Lens	LVMI Fluor		MI Fluor	
Mag.	4x	2x	4x	20x
NA	0.3	0.14	0.28	0.45
WD	6 mm	12 mm	10 mm	5.5 mm

Tab. 3: Immersion objective lenses by LaVision BioTec.

Web pages
Several authors have placed additional information into the web. Please refer to following pages for further assistance:
http://idisco.info/
http://clarityresourcecenter.org/
http://cubic.riken.jp/
http://www.chunglabresources.com/sw1
https://sites.google.com/site/seedbresources/

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