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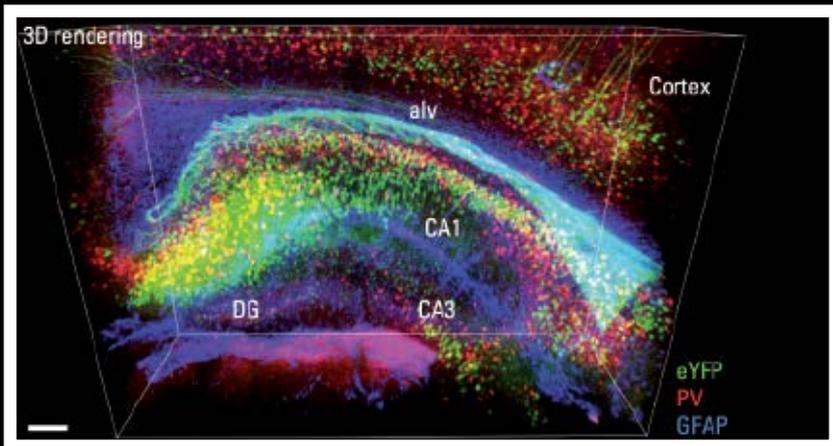
Dr. Rolf T. Borlinghaus, Dr. Andrea C. Mülter

Clearing Procedures for Deep Tissue Imaging

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Why clearing? Curiosity is human nature. And nothing attracts as much curiosity as the inside of living organisms. While in ancient times those who cut human bodies open to do research were put to death, and modern anatomy started only after Pope Clement VII allowed dissection [1], we can now watch brains working in living animals [2] – and have a good chance of soon being able to interfere with the observed activities for healing (or control) purposes.

The medieval ban on opening the human body was – at least in fiction - bypassed by the very special ability of Superman: x-ray vision. Although there has been some scientific discussion on that subject [3], it is clear that there is no way of looking through opaque material by visible light. Observation of tissue in living materials, e.g. through cranial windows to the brain, does not allow us to look too deep inside, as scattering and absorption still occur in the brain tissue. As an option for fixed materials, various protocols for "clearing" have been proposed during the history of microscopy. These protocols aim to both reduce scattering and to remove absorbing substances.

Clearing Approaches

Classical "brighteners" in microscopic preparation protocols are chemicals like potassium hydroxide and lactic acid, which modify the chemistry of the tissue. Especially chitin-containing objects benefit from such treatment. Clove oil was also used for making tissue transparent, it is easily miscible with Canada balm and has a similar refractive index (1.54). Besides bleaching absorbing molecules, the main problem in imaging thick biological objects is scattering, which occurs at the many boundaries of changing refractive index (fig. 1b & c) through which the light has to travel. Consequently, the major task of clearing methods is to "equalize" the refractive index without destroying the three-dimensional structure and without degradation of possibly present fluorochromes.

One option for reducing the refractive index variations in tissue is to remove the water and replace it by an organic compound that has a higher refractive index. As long as 100 years ago, Spalteholz described such a treatment with benzyl alcohol and methyl salicylate [4]. He proposed

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that the final mounting solution must have the same refractive index as the average index of the desiccated material. Many derivative procedures have been published since, which mainly vary in the chemicals applied (including clove oil). All these protocols have two common steps: first "dry" the sample by incubation with increasing concentrations of ethanol or tetrahydrofuran [5] etc., then refill with high- index solvent like glycerol, benzyl alcohol-benzyl benzoate (BABB) or dibenzyl ether (DBE). Immunolabeling is not possible after such processing and must be applied before clearing. Fluorochromes are not very stable and imaging should be performed soon after the clearing. For most recipes, the sample will usually shrink during the treatment, which indicates that the three-dimensional structural coherency might be partially lost.

Similar approaches try to increase the refractive index of the aqueous phases by adding water-soluble compounds, such as glucose, fructose (SeeDB [6]) or urea (Scale [7]). In this case, the sample does not need to be desiccated. The volume changes depend on the method and can be low (SeeDB) or large (Scale). Due to the high osmolarity, the material may expand by a factor of 1.5 and more along one axis. Just the expansion might be a major contribution to higher transparency. In order to look a far distance down into the tissue, expansion of the sample is counterproductive, as the free working distance of the objective lens is a limit for imaging depth. If a sample expands 1.6-fold, the effective maximum imaging depth is reduced by 38 %.

A third way of reducing refractive index variations is to replace the water ($n=1.33$) by polar solvents with higher refractive index, ClearT [8] uses incubation with Formamide ($n=1.45$). This procedure does not change the volume too significantly and
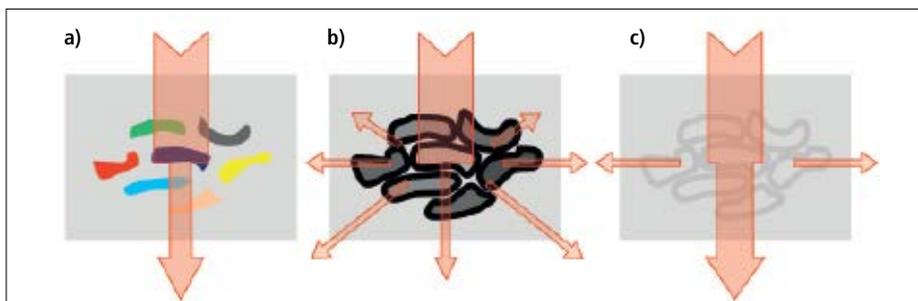

Fig 1: a) Low transparency through absorbing pigments b) Low transparency due to effective scattering at surfaces with changing refractive index c) High transparency after equalization of refractive indices.
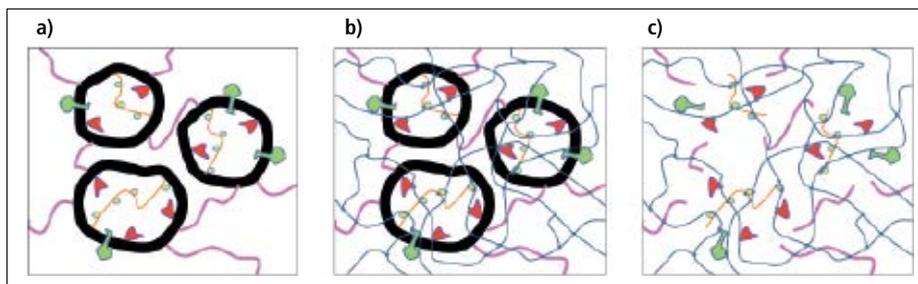

Fig 2: a) Biological tissue with membranes (thick black lines) supporting the spatial distribution of proteins (colored) b) After perfusion with polymer components, the proteins are pinned to a hydrogel (thin blue lines) with crosslinks (blue dots) c) After removal of the membranes, the tissue is transparent and even permeable to macromolecules, e.g. antibodies and fluorochromes. The proteins and other biomolecules are fixed to their *in-vivo* coordinates.

allows lipophilic tracer dyes to be imaged, as the protocol does not use detergents which disrupt the membrane structures. A variant adds Polyethyleneglycol, which protects green fluorescent protein from disintegration und hence produces better signals in samples with fluorescent protein labels.

Pellucidation Procedure

In his book, Spalteholz used the term "das Durchsichtigmachen" – a strange word, even for Germans. It is more specific than "clearing" as it literally means "pellucidation" i.e "make transparent" - of course without destroying the spatial structure. To achieve this goal by an entirely different approach, the group of Karl Deisseroth set out to develop a new procedure to really make brains transparent without destroying the protein assembly, and with the option to use serial immunostainings for investigating the molecular arrangement of functional meshworks. They called their method CLARITY [9].

As mentioned above, the troublemakers for deep imaging are the lipids. The membranome of a brain is a complex and densely stacked conglomerate of soma membranes, axonal and dendritic tubes, nuclear envelopes, compartmental membranes and synaptic structures. On top, this ensemble is coronated by an enormous number of vesicles – very effective scatterers – moving about in all cells. The job of all these membranes is to keep the structure intact and separate the various compartments. Just adding detergent would dissolve the membranous structure and convert a brain into something close to primordial soup.

The first step in the CLARITY protocol (fig. 2) is therefore a procedure that pins the proteins to their 3D coordinates – without relying on the membranous structures. To that end, a whole (anesthetized) animal is perfused with a monomer that is used to produce hydrogels, classically acrylamide, and a cross linker (formaldehyde). The formaldehyde will covalently bind to proteins and other biomolecules, which is its classical function as fixation agent. It will also crosslink the hydrogel monomers to the biomolecules, but not to lipids. The solution also contains a thermal initiator. After thorough perfusion at low temperatures, the temperature is raised to 37 °C. The added thermal initiator starts the polymerization, and a hydrogel is generated – just like any acrylamide gel. Most of the proteins become part of the three dimensional mesh, which is therefore called a

Fig 3: The newly developed HC FLUOTAR L 25x/1.00 IMM (n_e=1.457) motCORR VISIR objective for specimen treated with CLARITY.

hybrid gel. This is how the proteins are pinned to their *in vivo* locations.

In the second step, the brain is incubated with an SDS solution. The detergent will dissolve the membranes and form micelles. The hydrogel, including the bound biomolecules – is not affected. To speed up this clearing process, the removal of the micelles is accelerated by electrophoresis, hence the procedure is called electrophoretic tissue clearing (ETC) – the lipids are electrically sucked out of the brain. The remaining structure is a membrane-free hydrogel that contains most of the proteins at their original positions. The optical properties have completely changed: the hydrogel is highly transparent (soft contact lenses are made of hydrogels) and the refractive index is comparably homogeneous throughout the whole brain. Consequently, scattering is close to zero, and the tissue is pellucid.

The whole procedure is very friendly to fluorescent proteins. If the animal contains one or a series of fluorescent proteins, the cleared tissue will render these proteins at the original locations, and the fluorescence is not quenched (which is a problem in BABB preparations, and partially also in Scale protocols). The sample is not only transparent, but also allows diffusion of macromolecules into deep layers. Serial immunolabeling with a set of differently colored fluorochromes becomes available for whole brains. This is the key for structural brain mapping projects and similar research tasks. Lipophilic tracer dyes, such as DiI, will of course not work with lipid-free samples (unless one uses dyes that can crosslink with the hydrogel).

Optics that Fit

The samples prepared with the CLARITY method may be imaged with all fluores-

cence techniques, preferably with confocal microscopes, which offer the required high axial resolution. The lenses should have a sufficiently long working distance to allow focusing through the brain without mechanical slicing. For brain mapping, the field of view should be large, in order to record a full brain horizontally in as few as possible tiles. The spatial resolution required will call for high numerical apertures, and multi-channel multiphoton imaging would need high performance in a spectral range from blue to near IR. A key to having all these parameters properly even at a depth of several millimeters is a control for the optical path length, i.e. a correction collar at the lens that allows it to be adapted to different refractive indices.

Leica is releasing such a lens: HC FLUOTAR L 25x/1.00 IMM (n_e=1.457) motCORR VISIR. This lens offers 22 mm field of view at 25x magnification, i.e. a field of nearly 1x1 mm. The lateral resolution is some 250 nm for blue-green light, and the free working distance is 6 mm. This is sufficient to sample an object of 12 mm thickness, when turned once. The lens has a high transmittance from 470 nm to 1200 nm, and it is designed for samples with refractive indices around 1.45. The correction collar is motorized and software- controlled, this allows automatic correction while focusing into different positions in the sample.

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Optics for CLARITY by Leica Microsystems – Specifications

VISIR Correction for Confocal and Multiphoton Microscopy

The broad spectral correction of the objective ensures outstanding imaging performance with single- and two-photon excitation. Color correction of this objective is complemented by the range of refractive index correction available from the motorized correction collar, to produce high axial and lateral colocalization in specimen cleared with the CLARITY method and mounted in FocusClear®.

Maximum Transmission in VIS and IR for Brighter Images

Superior anti-reflective coatings make the Leica HC FLUOTAR L 25x/1.00 IMM motCORR VISIR highly transmissive (>85%) from 470 to 1200 nm. This maximizes the number of photons for excitation and results in brighter images from deeper tissue sections. Efficient collection of emitted photons reduces the need for high laser powers and protects the specimen from photobleaching.

Transmission [%] vs Wavelength [nm] — HC FLUOTAR L 25x/1.00 IMM motCORR VISIR
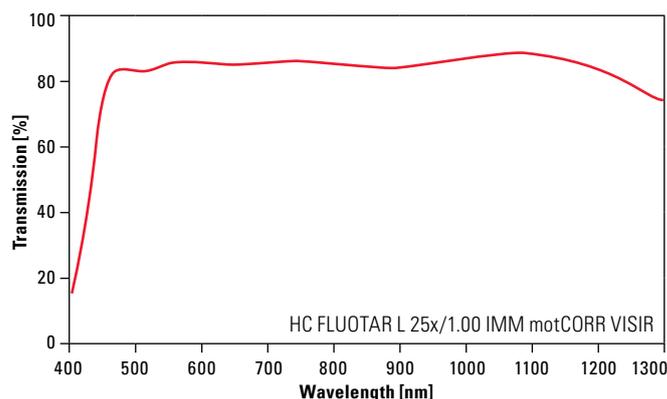
DEEP TISSUE IMAGING OF CLARIFIED SPECIMENS WITH SINGLE-PHOTON AND TWO-PHOTON MICROSCOPY

Imaging Technique	General Principle	Advantages	Limitations
Single-photon confocal microscopy	Confocality achieved by removing out-of-focus light with confocal pinhole	Broad range of fluorescent labels suitable for multi-color imaging.	Photobleaching of the whole tissue by out-of-focus excitation light. Performance dependent on dispersion of specimen.
Two-photon microscopy	Excitation by two photons of approximately twice the wavelength – and half the energy – necessary for one-photon excitation. No pinhole required.	Photobleaching restricted to the focal plane. Highly efficient detection with non-descanned detectors. Increased depth by reduced scattering of infrared excitation light.	Not all fluorescent labels appropriate. Pulsed infrared laser required.

SPECIFICATIONS OF LEICA HC FLUOTAR L 25X/1.00 IMM (N_E=1.457) MOTCORR VISIR

Magnification	25x
Numerical Aperture	1.0 in n_e=1.457
Free Working Distance	6 mm
Correction Collar	Yes, motorized with full control via LAS AF software
Objective Thread	M32 for use with single objective holder on Leica DM6000 FS and CFS
Field of View	22 mm diagonal in intermediate image plane with Leica TCS SP8 1800 Hz scanner

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