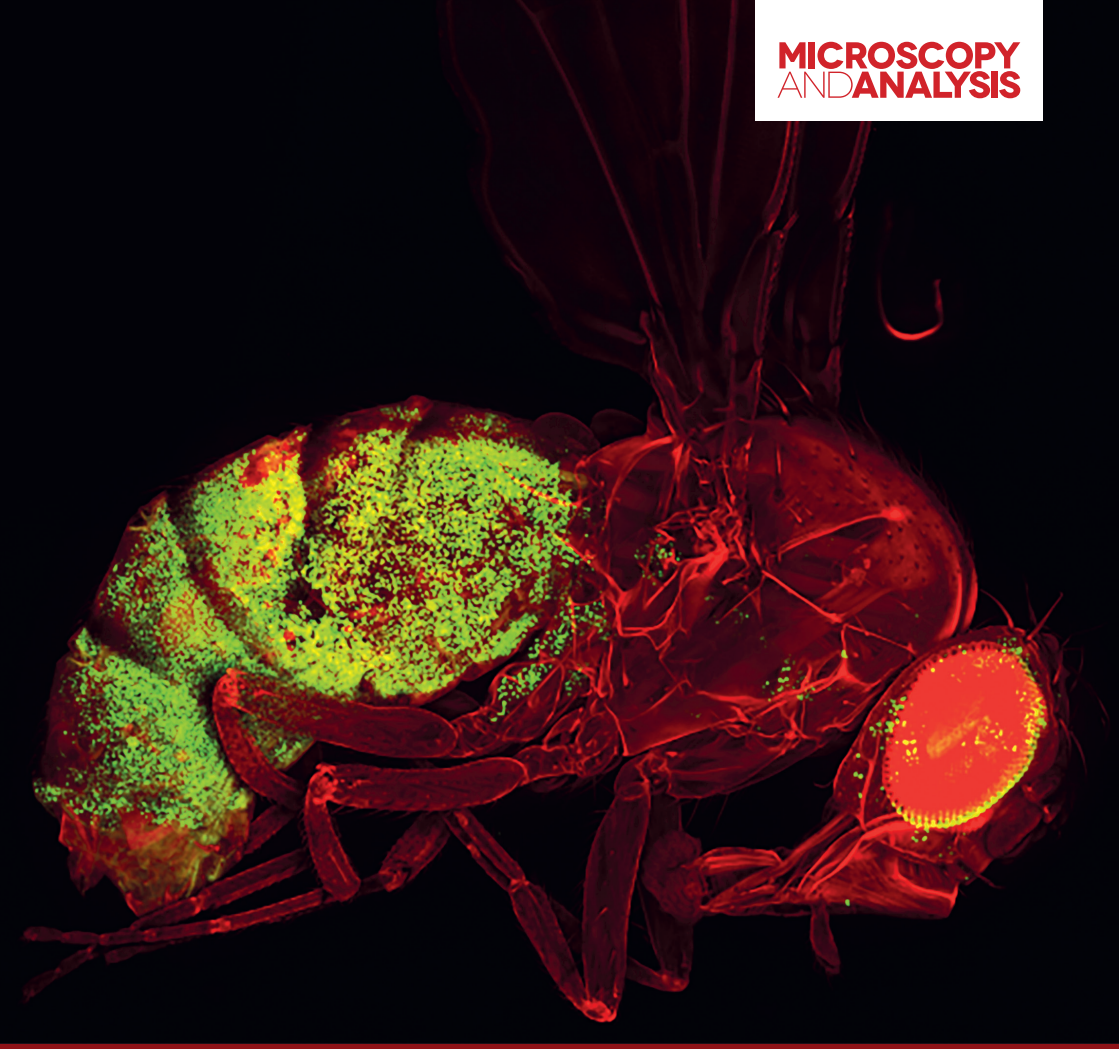
MICROSCOPY AND ANALYSIS



The Art of Tissue Clearing

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Essential Knowledge Briefings

First Edition 2021

Cover image: *Drosophila melanogaster*. Courtesy of Daniel Reumann and Jürgen Knoblich.

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PREFACE

The purpose of this ebook is to provide the interested reader with insights and theory on optical tissue clearing. It should serve as a starting point to explore this rapidly expanding technology. The author does not intend to replace any of the excellent and comprehensive reviews that recently appeared on this subject, nor to provide protocols or technical details on specific techniques.

Optical clearing is needed to tackle the problem that biological tissue isn't naturally transparent. Beyond a certain thickness, it quickly disperses the order of any light shone into it, obscuring internal features. For more than 100 years, scientists have developed a succession of optical clearing techniques for making biological tissue more transparent, so that these internal features can be studied with light microscopy. Such techniques not only need to be able to clear a wide range of different biological tissues, both animal and plant, but they ideally need to do so while preserving fluorescence signals and without altering the tissues in any way. This has required a lot of chemistry and quite a bit of optics.

The principles of optics encompass some complex matters that for non-physicists offer lots of reasons for losing their orientation in a confusing universe of physical laws and mathematical formulas that describe the behavior of light and its interactions with matter. One way to stay on track in this territory is to recapitulate how the theory of light and some of its technological and scientific applications have evolved over time, which is how we begin this book. Obviously, this approach must remain fragmental and incomplete here, but

should serve as a point of embarkment for the courageous.

Once we have got to grips with light and all its intricacies, we move on to a short history of optical clearing, showing how the basic approach to making biological tissues transparent has generally remained the same over time. This culminates in short profiles of some of the most commonly used clearing techniques, both solvent- and aqueous-based, for animal and plant tissues, and the latest microscopy technologies, including confocal laser scanning microscopy, light-sheet microscopy and super-resolution microscopy.

OPTICAL TISSUE CLEARING

"In the vast majority of cases tissues are too opaque for satisfactory examination until they have been treated with certain clarifying reagents or clearers which render them more transparent."

This assessment from 1906 by M. F. Guyer in *Animal Micrology*, who therein defines clearing as "the rendering transparent of tissue elements", summarizes quite well the state of the art of optical tissue clearing around the beginning of the 20th century. The author mentions "reagents such as glycerine" for objects to be cleared directly from water, while xylol is described as "perhaps the most useful and rapid clearer" for objects that have previously been dehydrated in alcohol. He concludes that "after tissues have been cleared" they must be mounted in a suitable medium for inspection.

Werner Spalteholz was a German anatomist at the University of Leipzig. (Image courtesy of the University Archive Leipzig)

The anatomist Werner Spalteholz (1861–1940), who is generally acknowledged as the pioneer of modern optical tissue clearing, must have embarked on his studies based on a similar knowledge. In his book *Über das Durchsichtigmachen von menschlichen und tierischen Präparaten* (*On methods of rendering human and animal preparations transparent*) from 1911, he wrote: "The experiments on which my method of producing transparent preparations

of whole animal bodies, body parts and organs is based, date back to 1906."

In patent application no. 229044, filed with the Imperial Patent Office in 1908, Spalteholz's methods and the motivation for his studies are nicely described in detail: "With the methods commonly used for the production of microscopic preparations, it was not possible to make the construction of organized bodies sufficiently accessible for observation... By cutting into sections, many parts are destroyed and the connection between the parts is broken. Cutting up into sections promotes the recognition of details but prevents the recognition of the overall arrangement."

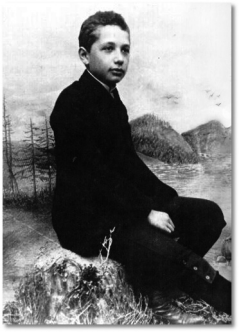
Since Spalteholz, the term optical tissue clearing has referred to procedures that render opaque specimens transparent, so that, as far as possible, details can be observed without sectioning. His patent also summarizes his methods for obtaining transparent samples: "It has been recognized that a body can only be made transparent if it is saturated or filled with substances whose refractive indices are as close as possible to that of the body to be made transparent or translucent."

This crucial statement holds true until today and will be discussed in detail below. Before we look at Spalteholz's techniques and their modern variations more closely, however, let's take a glance at the theory of light and its interactions with matter. What is it that makes matter either opaque and unclear, or translucent and sometimes so transparent that it becomes (almost) invisible?

Light and matter

There are multiple ways to describe the phenomenon of light. Ray geometry can help to construct complex optical instruments such as telescopes, camera objectives and microscopes. In microscopes, wave functions are needed to describe how light originating from a point on a specimen and travelling to a detector smears out into a three-dimensional volume that is accurately outlined by what we call the point spread function (PSF). And finally, within this light path, we are confronted with phenomena such as fluorescence or light detection, which leave us with no choice but to accept that light (under those circumstances) can best be described as a shower of tiny massless photons that hit matter (and the specimen in our sample chamber) at incredible speed.

"There is hardly a simpler law in physics," wrote Albert Einstein (1879–1955), "than that according to which light is propagated in empty space." Einstein's claim still holds true today, but as soon as matter becomes involved things get complicated. For users of a microscope, the interplay of light and matter within the optics and the specimen is both an opportunity and a challenge.



Albert Einstein at the age of 14, just a few years before publishing his groundbreaking thoughts on light quanta. (Source: Wikimedia Commons)

Looking at an ideal vacuum under a microscope may be inspiring for specialists of quantum field theory, but the "structure of the absolute nothing", as David Tong, a specialist in quantum field

theory at Cambridge University, calls it (Tong, 2017), quite quickly becomes somewhat monotonous for most ordinary people. The moment we bring a specimen of matter into the microscope's light path, however, we must deal with a hodgepodge of interactions between the light and this matter. These interactions include various phenomena, such as absorption, emission, scattering, diffraction, refraction, reflection, polarization, and dispersion.

Some of these interactions are quite desirable, because without them matter would remain totally invisible and as monotonous as the perfect vacuum. But other phenomena can render specimens opaque or the beam path of the light so incomprehensible that the formed image no longer provides meaningful information about the sample. To understand the causes of such phenomena, and design and select strategies to deal with them, we must look at some aspects of the physics of light and its interactions with matter.

So, what is light?

Unfortunately, the answer to this question is complex and not as intuitively understandable as one would wish – some people think it's not understandable at all. Looking at some details of how the theory of light emerged will not solve this problem entirely, but at least it will show that the struggle to understand light has a long history.

Waves

René Descartes (1596–1650) saw in light a chain of instantaneous impulses through an invisible medium – the so-called

'æther'[1]. Descartes, who of course could not yet think of light as a non-contact action at a distance between two electrons, was puzzled by the question of how sunlight could affect his eyes through a solid pane of glass. In his publication *La Dioptrique* from 1637, the Frenchman vividly compared the glass matter with grapes in a wine press vat and the ether with the juice that flows around them.

Robert Hooke (1635–1702) suspected wave-like vibrations spreading through this æther, and Christiaan Huygens (1626–1695) formulated a comprehensive wave theory of light in 1678. According to Huygens' theory, every small place in a luminous object, be it the Sun, a candle flame, or a piece of glowing coal, should be the center of concentric circles representing expanding and overlapping waves. In his *Traité de la Lumière* Huygens argues that the particles of the æther were not arranged in straight lines, but wildly jumbled like balls on a billiard table, so that an impact of one ball would move the others randomly in all directions. Whether the metaphors of Descartes and Huygens reflect cultural preferences between French and Dutch scholars of the time remains to be unraveled

Isaac Newton (1642–1726), a professor in Cambridge at the time, was extremely displeased with such theories. In his view, waves could not provide a plausible explanation for the straightness of light propagation. Instead, he suggested in his own *Hypothesis explaining the properties of light*, that "light is neither æther, nor its vibrating motion, but some-

[1]In Greek mythology Æther was a personification of the upper sky in which light and the gods were seated.

thing of a different kind propagated from lucid bodies". Newton carefully specified this "something of a different kind" as a multitude of "unimaginable small and swift corpuscles of various sizes, springing from shining bodies at great distances one after another ... and continually urged forward by a principle of motion, which in the beginning accelerates them, till the resistence of the æthereal medium equals the force of that principle".

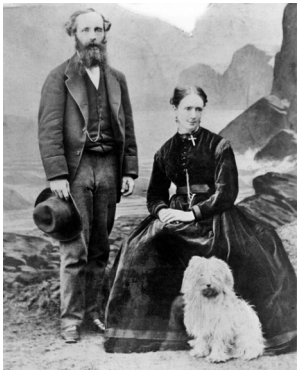
Newton's hypothesis is often regarded as the birth of a primordial particle theory of light. It seems worth mentioning, however, that the Roman scholar Lucretius Carus (99–55 BC) had already written: "The light and heat of the sun; these are composed of minute atoms which, when they are shoved off, lose no time in shooting right across the interspace of air in the direction imparted by the shove".

The criticism of the influential Newton, who apparently had a quite unfriendly relationship with Robert Hooke, made it difficult for the wave theory of light to develop further for an entire century. In 1802, however, Thomas Young (1773–1829) helped bring about a breakthrough with his famous double-slit experiment. Young vividly described a preliminary stage to his experiment in a presentation to the Royal Society in 1803: "I made a small hole in a window-shutter", he wrote, "and covered it with a piece of thick paper, which I perforated with a fine needle". Apparently, Young then used a mirror to direct a ray of sunlight through this pinhole onto the opposite wall and "brought into the sunbeam a slip of card of one-thirtieth of an inch in breadth and observed its shadow".

The groundbreaking conclusions Young drew from his

observations are more easily explained with the more sophisticated double-slit experiment. This experiment had two conditions: either light shone through two slits simultaneously, or one of the two slits was closed at a time.

Opening both slits resulted in a stripe pattern on a projection screen behind the slits, which could not be explained by a simple superimposition of the patterns that were seen when light was shone through each slit individually. On the contrary! In some regions of the projection pattern, the light was intensified by opening the second slit (as expected), but in other places, it became darker. It was as if the light coming through one slit would extinguish some of the light coming through the other slit. This could not possibly be explained by the stream of corpuscles suggested by Isaak Newton (and Lucretius Carus). Instead, the result was regarded as proof of the wave nature of light, because only the assumption of destructive interference of two waves made this observation plausible.



James Clerk Maxwell and his wife Katherine

Then, Michael Faraday (1791–1867) discovered the induction of electricity by a moving magnet and a link between electromagnetism and light. This made the wave theory of light unstoppable. It culminated in 1864 in the *Dynamic Theory of the Electromagnetic Field* by James Clerk Maxwell (1831–1879), who succeeded in combining all factors of Faraday's observations in a single

field theory – the famous 'Maxwell equations'.

Maxwell's equations describe exactly where and when electric or magnetic forces act when an electric charge or a magnetic dipole is moved somewhere in space and time. With his theory, Maxwell could also describe phenomena of optics "which were almost insurmountable for the theories of the elastic ether". In Maxwell's theory, light became a chain of electrical and magnetic energy fields linked by induction, which propagated at a finite speed in the form of a wave that filled space.

Photons

For some time, Maxwell's theory seemed flawless. Heinrich Hertz (1857–1894) later wrote: "When studying this wonderful theory, one has the feeling that these mathematical formulas have a life of their own and an intelligence of their own that far exceeds our own wisdom and that of their discoverer." But although conclusive, Maxwell's theory soon turned out to be incomplete for describing the nature of light. The wave theory of light was now able to describe all phenomena of optics, but it led to "contradictions with experience" in the case of phenomena of "light generation and light transformation".

Albert Einstein provided some intuitively understandable arguments in a groundbreaking publication in 1905, where he pointed out that there is a "profound formal difference" between the physics of matter and the physics of empty space. The energy state of matter can be completely described by the sum of all the states of a finite number of atoms and

electrons that cannot be further divided, while space, on the other hand, can grow continuously into ever larger dimensions. "According to Maxwell's theory of light, the energy of a light beam emitted by a point-like light source is" thus "continuously distributed over an ever-growing volume" and the energy density in space must therefore continuously decrease. Einstein recognized that this has fundamental consequences.

When Philipp Lenard (1862–1947) had irradiated a metal surface with UV light, he observed the release of free electrons from the metal, a phenomenon called the 'photoelectric effect'. Einstein argued that, following Maxwell's theory, the energy density should continuously decrease the further away the irradiated metal surface is moved from the light source, if the intensity of the light source is kept constant and dim. Consequently, no signal should be detectable at more than a certain distance, because the available energy would then be so spread out in space that its density would no longer be enough to perform the work necessary for releasing an electron from the metal. This, however, was not in agreement with the actual observations. Electrons were still released, but instead of disappearing entirely, these events became rarer and rarer the further away the light source was moved from the metal surface.

Max Planck (1858–1947) had already found a few years earlier that the energy that can be transmitted by an electromagnetic radiation has a minimal value $E = h \cdot \nu$, but he refused to accept the consequences of his own discovery. Einstein, however, ingeniously concluded from his simple observation that "when a beam of light emanating from a

point is propagated, the energy is not continuously distributed over larger and larger spaces, but rather consists of a finite number of energy quanta located in points in space, which move without dividing and can only be absorbed and generated as a whole". This was the birth of light quanta, which Gilbert N. Lewis (1875–1946) later (in 1926) called 'photons' and which we nowadays shoot through our samples so generously, as if they were nothing special.

The birth of light quanta ushered in a wave/particle duality that continues to challenge our imagination today. Both theories – Maxwell's equations and Einstein's light quanta – are still valid and fundamental. They coexist but have proven difficult to reconcile. How could a wave be a particle? Or even worse, how could a particle be a wave at several places simultaneously? Richard Feynman (1918–1988) described the shock triggered by Einstein's quantum theory with the words: "Wave/particle duality was a phrase used to describe a state of confusion."

However, this confusion turned out to be constructive. As early as 1909, G. I. Taylor (1886–1975) repeated Young's double-slit experiment with very-low-intensity light. He was able to show that the interference of light from both slits also occurred when the photons seemed to "fly" individually, and at long time intervals, from the source through the slits to the plane of projection. How was this possible? What did it mean? How could a photon passing through one slit know whether the other slit was open or closed?

Even the cleverest minds of their time despaired of such questions. Einstein wrote the much-quoted words to a friend:

"All the fifty years of conscious brooding have brought me no closer to answer the question, 'What are light quanta?' Of course, today every rascal thinks he knows the answer, but he is deluding himself." And his younger colleague at Princeton University, Richard Feynman (whom I think Einstein might have meant by "rascal", because he didn't like Feynman's introduction of probability into the physics of light), summarized his understanding of light with the famous sentence: "I think I can say with certainty that nobody understands quantum physics."

And today?

Let us return to the present from this fragmentary excursion into the history of theories of light with a conclusion from a contemporary review on *What is a photon?* by Vasant Natarajan: "Certainly, the present model of the photon and radiation has many puzzling features that make it unsatisfactory. To paraphrase Einstein, perhaps we are deluding ourselves into thinking that we know the photon" (Natarajan, 2013).

With that modesty in mind, it seems (for our purpose) best to stick to a simple but sufficiently comprehensive interpretation of the photon. Let us simply regard it as an uptake of energy by an electron (the absorber) and let us assume that this energy must have been taken away from another electron (the emitter). Anything else would contradict the first law of thermodynamics, according to which energy cannot be generated *de novo*, but only be transferred and converted.

But this raises the question of what happens between emission and absorption: is there even a photon that exists independently after emission? And if so, where is it located? What does it do? What path does it take?

Hugo Tetrode (1895–1931), and later John Wheeler (1911–2008) and Richard Feynman, formulated a theory according to which light is a direct long-distance effect between emitter and absorber without any intermediate field. Within their theory, an independently existing photon would be as absurd as an empty bar full of conversation (Natarajan, 2013).

This assumption is powerful, because it allows us to gnore the voyage of the photon from the emitter to the absorber. We therefore avoid the need to understand this voyage, which neither seems possible, nor is necessary. Where the photon is located after its emission is not defined. Only the probability by which it can be observed at a certain place and at a certain time is mathematically describable by a wave function. The exact shape of this wave function depends on the amount of energy transmitted and on all the matter in the space under observation.

The probability density of photons in space and time, which are described by this wave function, has no significance for a single photon. Thus, at this point, it is very important to clearly separate a photon from light! Photons are the building blocks of light, but light can only be understood as the collective behavior of all its photons together. It is impossible to create a meaningful optical image with a single photon, because from where a photon is detected, no conclusion can be

drawn about its origin. Photons have no age and no individual identity. If, on the other hand, a very large number of photons are considered over a certain period, the probability density of photons corresponds exactly to the relative light intensity observed.

In summary, many optical phenomena, including refraction, diffraction, scattering, dispersion, opacity, and transparency, can adequately be described as a wave function of space and time, quantifying the probability density of all photons involved in the observed phenomenon. Metaphorically, photons constitute light like letters constitute lyrics. There is no light without photons and no lyrics without letters, but a bag full of letters or photons are neither lyrics nor light.

Translucency and transparency

Broadly speaking, optical clearing refers to a collection of methods for making opaque things transparent. Matter is intuitively associated with visibility. Things are usually visible. We tend to only believe what we see, and thus we are shocked when we bump into a perfectly cleaned pane of glass that was invisible until we feel the impact.

Similarly, when we look at the twinkling stars in a clear night sky, it can be easy to forget there is at least 100km of atmospheric matter between us and outer space. This matter is not visible, but we know that it exists. So, while invisible matter does not cause us lots of headaches in everyday life, the theory behind its flawless clarity is less intuitive than we might expect.

To approach this complex topic, ordinary frosted and clear electric light bulbs may serve as 'model organisms' for deciphering the transition from opacity to transparency. Methods that were invented 100 years ago for the industrial production of these commonplace items nicely illustrate the fundamentals of opacity. They show how transparency can be created and how opaque objects can be cleared.

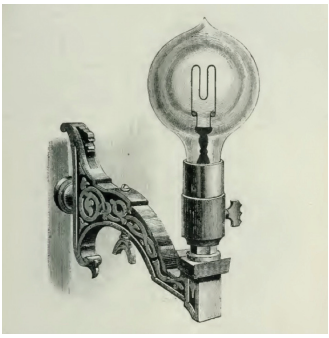
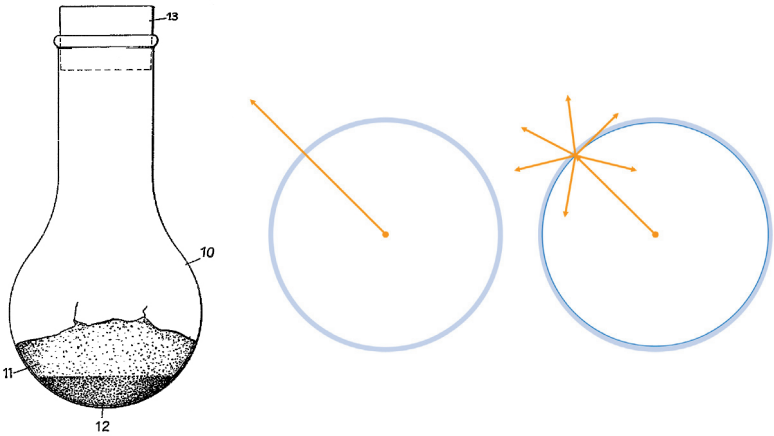


Illustration of a light bulb in the official catalogue of the International Electricity Exposition in Paris (1881).

In 1881, Thomas Alva Edison (1847–1931) used electric light for the first time to illuminate his pavilion at the Exposition International de l'Électricité in Paris. His light bulbs consisted of a clear glass sphere, in the center of which a white-hot filament served as a source of photons. To reduce the blinding light intensity of this filament by dispersing it evenly over a larger surface, the outside of the glass ball was frosted by etching the glass with hydrofluoric acid.

Several contemporary patents explain how and why this frosting later moved from the outer to the inner surface of the bulb, where it remains to this day. "The desired luminous effect of the light source," claimed Berlin inventor C. B. Herrmann in 1912, "distributed over the entire glass jacket, is achieved by this outside frosted bulb, but the evil must be accepted that such frosted glass lamps lose their light transmission over time, because the matt (roughened) outer surface of the lamp easily allows dust and impurities to accumulate."



A figure from 1925 illustrates how the matting was then applied inside the bulb. The internally roughened glass bulb (10) was filled with pigment powder (12). The bulb was then closed by a stopper (13) and the powder distributed by shaking. Another patent from 1927 explains that "to produce a milk-white appearing, light-scattering bell, a coating of silicon dioxide" or other inorganic chemicals were suitable. A reddish coloring of the bell could be achieved with a coating of iron oxide, while a greenish coloring could be accomplished with a coating of chromium oxide.

At first glance, the reasons why glass bulbs treated in this way become turbid seem obvious. Rays of light appear to leave the light source in the center of the bulb and pass through the clear glass bell undisturbed, in a straight line. Thus, the lamp filament is clearly visible. In the frosted glass bell, the light beam hits pigment particles on the inside of the bulb and is scattered by them in all directions. Such coated bulbs allow the same spectral range of light to pass, if the coating material

has the same absorption characteristics as the glass itself, but despite their high translucence, the coated bulbs are turbid.

When looking at this situation at the level of photons, however, things become complicated. Each photon that leaves the light source in the two bulb types hits a suitable electron in either the clear or the frosted glass, by which it is absorbed and re-emitted instantly. This 'collision' is like an elastic impact in classical physics, but the directions of neither the impacting nor the recoiling photon are defined (as discussed above) and are therefore random. In both glass types, photons are thus scattered in all directions.

This is surprising, because what is it then that creates the difference between the clear and the frosted bulb? What mechanisms make the coated glass turbid? Or, more importantly in the context of our topic here: what makes the uncoated glass bell so perfectly transparent?

To explain this, we must apply the wave theory of light. The probability density of a photon propagates from the moment of its emission as a spherical wave through the vacuum inside the glass bell, with the emitting electron located at the center of this sphere. As soon as the wave reaches the inner surface of the glass bulb, all suitable electrons in the glass matter along the wave front become starting points of secondary spherical waves, which also represent the probability density of the recoiled photon. All these waves now interfere with each other and with the primary wave.[1]

[1]Einstein's arguments from above should be recalled here: The wave front expands to a spherical diameter of 300,000 km *(continued overleaf)*

There are several million silicon dioxide molecules in a small glass cube with an edge length in the range of the wavelength of visible light (e.g. $\lambda = 500nm$). Therefore, in the plane of the incident primary wave, each electron will always have adjacent neighbors in all directions at distances of half the wavelength (i.e. 250nm). The secondary waves emerging from such pairs of electrons will interfere with one another. Parallel to the plane of the primary wave (i.e. in the lateral direction) this interference is destructive, because the waves cancel each other out. Perpendicular to the plane of the primary wave (i.e. straight out of the bulb) the interference is constructive. In the forward direction, the waves therefore reinforce each other and overlap with all the other secondary waves to form a new spherical wave front, which, by constantly repeating this process, moves outwards through the glass, almost as if it didn't interact with the bulb at all. This creates the clarity and transparency of the unfrosted bulb.

The above-described process breaks down in frosted bulbs. Here, the primary wave first hits the fine layer of silicon dioxide particles applied in the frosting process. The electrons of these particles now also serve as starting points of secondary spherical waves, but due to the inhomogeneity of the particle layer, there are often no adjacent electrons at a distance of $\lambda/2$. This means the destructive interference to the

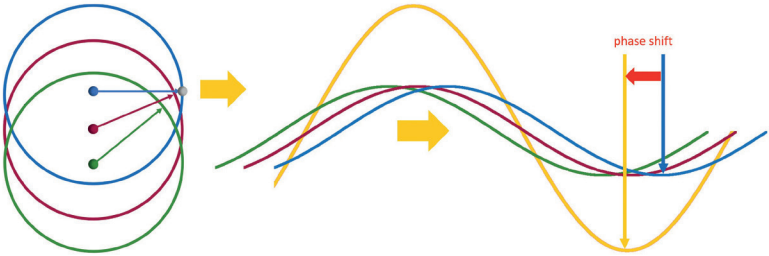
(Continued from page 21), in one second, which makes the absolute probability of finding this one photon in each volume the size of an absorbing electron extremely small. However, this relative probability density still matters, because equal probability densities result in equal light intensities when many photons are involved.

side, as well as the constructive interference to the front, occur at much lower efficiencies. The light thus remains randomly scattered in all directions, making the glass bell appear milky and unclear.

Thus, a high density and homogenous distribution of light-scattering electrons is crucial to the transparency of matter. Inhomogeneities in the optical density of a sample, caused by an inhomogeneous distribution of light-scattering electrons, turns the sample turbid and opaque. Such inhomogeneities can be caused by clearly defined domains, such as air bubbles, lipid droplets, membranes, organelles, and large protein or carbohydrate complexes in biological tissues (e.g. fixed mouse brains or plant roots).

Refractive index

The so-called refractive index (n) is a useful parameter to describe and quantify the density and distribution of light-scattering electrons in a sample. The refractive index of any kind of matter is defined as $n = c/c_m$, the quotient between the speed of light in a vacuum (c) and the speed of light in the specific matter (c_m). In matter with a refractive index of n = 2, light would thus travel at half the speed compared to a vacuum. This reduced speed of light (c_m) does not refer, however, to the propagation of the individual photon, which still travels at the full speed of light c from electron to electron. Rather, c_m refers to the velocity of all the interfering waves combined while travelling through the matter.



Schematic illustration of the slowing down of light in media with high optical density, exemplified by one electron (blue) and its two neighbors (red and green). While photons 'jump' from electron to electron at the full speed of light c, the resulting 'light wave' (orange) travels more slowly due to the consecutive phase delays. The refractive index n is a measure of the resulting slowing of light in media relative to a vacuum.

This is another example of a phenomenon that can be explained by regarding light as waves. A simplified two-dimensional model illustrates the situation. When a plane primary wave hits a line of scattering electrons (blue, red, green), they will simultaneously send out secondary waves that constructively interfere forwards. The next available electron (grey) in the forward direction will first be reached by the wave front from the closest emitter (blue), followed by the more distant ones (red and green) with a small but gradually increasing delay.

The constructive interference of the three primary waves (blue, red, green) will increase the amplitude of the resulting (orange) wave but shift its phase backwards (orange vs blue curve). So, while all primary waves still expand at the speed of light c, the phase of the resulting secondary wave will be shifted backwards at each light-scattering electron, thus reaching the next light-scattering electron with a small but not negligible delay. The total delay depends on the distance that is traversed in the medium and on the density of light-scattering electrons therein.

Biological samples possess a high diversity of cellular constituents, which all contribute to the turbid and milky appearance of the samples.[1] Three cellular components are mainly involved in this effect: lipids (n ~ 1.45), proteins (n ~ 1.6) and the aqueous cytoplasm with all its soluble constituents (n ~ 1.34). These components are dynamically organized in an interwoven three-dimensional network, together with many other components. The samples will thus be comprised of numerous coalescent microdomains of different refractive indices, at the borders of which the light will be scattered in all directions.

"*Any tissue with a homogeneous refractive index will be transparent. In the strictest sense, this requires that either the tissue is made up of only one component, or that it is made up of different components that all have the same refractive index.*" (Johnsen & Widder, 1999).

All methods of optical clearing therefore aim at creating a high density of homogeneously distributed light-scattering electrons – or in other words, a high and even refractive index – to achieve sample transparency.

[1]An exception is provided by marine zooplankton, which systematically use transparency for camouflage.

METHODS OF OPTICAL CLEARING

The milky, turbid, and opaque appearance of translucent specimens depends on the inhomogeneous distribution of light-scattering electrons, or, in other words, on numerous and irregularly arranged transitions between domains with different optical densities (refractive index mismatches). Roughly, three types of such domains can be identified: aqueous (extracellular liquid and cytosol), lipids (cell membranes, organelles, lipid droplets) and proteins (of numerous kinds). The optical density of these domains strongly depends on the exact local composition and distribution, and cannot be defined precisely. They range around $n \sim 1.34$ for aqueous domains, $n \sim 1.45$ for lipid domains and $n \sim 1.6$ for protein domains.

Optical clearing methods generally aim at tuning the refractive index of the sample close to the average refractive index of all sample components. Unfortunately, the range of optical densities in a sample is often too broad to find a useful average for such 'refractive index matching'. Most optical clearing strategies therefore rely on selectively removing all domains that seem unimportant for the actual examination, and subsequently matching the refractive index of the entire sample to the average value of the remaining domains (or higher). Notably, this step offers many possibilities for developing and optimizing specific protocols.

In an overview of plant-clearing techniques, botanist Rhys Owen Gardner defines the principle of clearing with the following words: "A piece of plant is cleared when some of its components are made visible at the expense of others while the form of the material remains more or less unaltered."

(Gardner, 1975). This might be a good point to mention that, depending on the kind of sample (and particularly true for plants), decolorization can be an important step to increase the translucency of specimens. Also, preventing colorization by the clearing process can be an issue, e.g. browning caused by Maillard reactions when exposing a sample to concentrated sugar solutions at elevated temperature. Vice versa, it is equally important not to destroy deliberate staining of the sample (e.g. fluorescence) by the process of clearing. These aspects will not, however, be covered in further detail here.

Three principal strategies for homogenizing optical density in specimens have been pursued:

1. Dehydration (removal of the aqueous domains), followed by delipidation (removal of the lipid domains) and subsequent refractive index matching to the protein domains (e.g. Murray's Clear, 3DISCO).
2. Raising the refractive index of the aqueous domains by immersion in water-soluble refractive index matching agents (e.g. Glycerol, TDE, Fruit).
3. Lowering the refractive index of the protein domains by hyperhydration, with or without delipidation.

Since these steps often lead to a significant destabilization of the sample, some protocols apply measures for stabilizing sample integrity, such as hydrogel embedding.

Werner Spalteholz

More than 100 years ago, the anatomist Werner Spalteholz published and patented techniques to render biological samples transparent. His descriptions are amazing to read

and parts of them still sound surprisingly up-to-date.

Spalteholz was interested in the three-dimensional reconstruction of heart vascularization, particularly of fine capillaries that could not be resolved by X-ray imaging. From his previous work, he knew that muscle and skin tissues become transparent when soaked in xylene (n = 1.497) after dehydration in ethanol. This method is still commonly used today, e.g. when mounting stained histological sections under coverslips.

When Spalteholz tried to clear heart tissue in xylene, however, this approach was not very successful and worked only for small pieces of tissue. For larger parts or entire organs, it completely failed. He speculated that pure benzol might improve the clearing, because of its slightly higher refractive index (n = 1.501). The results were indeed better, but the improvement was only mediocre. He therefore moved on to carbon disulfide (n = 1.628) and other liquids with an even higher refractive index, but to his surprise this rendered the samples completely opaque.

Werner Spalteholz in one of his seminars at the University of Leipzig. (Photo: Universitätsarchiv Leipzig)

As Spalteholz pondered this observation, he had a simple but ingenious idea: he transferred the sample into benzol and stepwise added carbon disulfate to increase the refractive index gradually, until the optimal clarity of the

sample was reached. This strategy was successful, and he recognized that there was no perfect mixture for all tissues from different organs and species, but that instead the refractive index of the clearing reagent had to be optimized for each sample.

"An (animal or vegetable) body reflects the least light and achieves the greatest possible transparency when it is saturated (and surrounded) by a substance whose refractive index is equal to the body's mean refractive index," he concluded.

In his initial method, however, Spalteholz perceived several drawbacks. First, carbon disulfide had a horrible odor and was extremely toxic. The addition of peppermint oil improved the smell but made the toxicity even worse, because the alarming smell was no longer present. Furthermore, carbon disulfide turned out to be very unstable over time. This not only changed its refractive index but also caused the elemental sulfur to separate out, thus producing a very fine deposit that turned the samples opaque.

"So, I turned my attention to substances with a refractive index equal to or higher than that of the carbon disulphide–benzene mixture, with the intention of producing liquids with a lower index by adding benzene," he wrote. "Extensive experiments now brought to my attention two liquids suitable for my purposes, the artificial wintergreen oil (methyl salicylic acid ester), with a refractive index of n = 1.534–1.538, and benzyl benzoate, whose index is n = 1.568–1.570."

With this, a protocol for optical clearing of intact biological specimens was born, which, with few modifications, is still frequently used today.

Organic solvent-based clearing protocols

All modern organic solvent-based optical clearing techniques are based on modifications of Spalteholz's original protocol. They all aim at creating refractive index homogeneity by three steps: 1) removal of water (dehydration); 2) removal of lipids (delipidation); and 3) matching the refractive index to the average refractive index of the remaining constituents of the specimen. While dehydration is usually achieved by an alcohol gradient, delipidation and refractive index matching are both achieved by infiltrating the specimens with organic solvents. The last step is crucial, because the 'remaining constituents of the specimen' and their average refractive index depend entirely on the nature of the specimen and therefore must be optimized individually.

Murray's Clear (BABB)

A first documented modification of Spalteholz's protocol was introduced in the 1980s by Murray and Kirschner, who were trying to see into *Xenopus* eggs. Andrew Murray (personal communication) recalls the invention:

"I was a postdoc of Marc Kirschner and we were discussing the problem of not being able to see into eggs. Marc hypothesized that the problem was the difference between the refractive index of the yolk platelets, which are essentially crystalline protein, and the cytoplasm that surrounds them, and suggested that matching the refractive index of the yolk platelets would stop refraction at the interface between them and the fixed cytoplasm. I then ordered a few compounds that were liquid and had what might be appropriate refractive indices. I

took eggs, homogenized them, dehydrated the diluted homogenates, resuspended them in the candidate compounds and used phase microscopy[1] *to decide whether the compound's refractive index was higher or lower than the yolk platelets. This revealed that benzyl alcohol (n = 1.5396) has a lower refractive index than the yolk platelets and benzyl benzoate (n = 1.568) has a higher refractive index. I just mixed the two compounds in different ratios until I found one that had the same refractive index as the yolk platelets: a 1:2 mixture of benzyl alcohol: benzyl benzoate.*"

'Murray's Clear' (also known as BABB) was born, but only reported later by Dent *et al*. (1989) for *Xenopus* eggs and embryos. Julien Barrere, a graduate student in Murray's lab, successfully applied the same chemical mixture to clusters of yeast cells. Orlich and Kiefer recently tested Murray's Clear on mouse embryos, describing only the cerebellum as retaining significant opacity. In their experience, the method was fast and well suited, although it induced significant tissue shrinkage due to the strong dehydration. One significant drawback of the method they found was fast and irreversible quenching of genetically encoded fluorescent proteins (Orlich & Kiefer, 2018).

(Note that many researchers share the assessment that BABB is a toxic and corrosive solution!)

[1]Phase contrast microscopy was invented in 1932 by Frits Zernike, for which he was awarded with the Nobel prize in physics in 1953. This method utilizes the above described fact, that the phase of a light wave is modified when traversing media of different refractive indices, to improve contrast.

THF/BABB

The observed quenching of fluorescent proteins (FP) is most likely caused by a disruption of the FP's secondary structure through the harsh dehydration procedure. Hans Ulrich Dodt's group therefore introduced tetrahydrofuran (THF) as a 'GFP (green FP)-friendly' alternative to dehydration by methanol or ethanol (Becker *et al.*, 2012).

3DISCO (THF/DBE)

Ali Ertürk further optimized this method for mouse brain by applying dibenzyl ether (DBE) as a clearing agent instead of BABB, obtaining better results, especially for genetically expressed fluorescent proteins (Ertürk *et al.*, 2012).

Ertürk remembers being confronted with a need for clearing when studying the path of regenerating axons in the spinal chord. This turned out to be difficult, because the spinal chord is quite opaque and the growing axons do not stay within a given plane. The observation that classical clearing methods relying on organic solvents were quite destructive to the fluorescent proteins expressed in these cells triggered Ertürk to screen various alcohols for dehydration that preserved fluorescence better than ethanol (Ali Ertürk, personal communication).

FluoClearBABB

Even better stability of fluorescent proteins over 3DISCO was reported by dehydration with either 1-propanol or tert-butanol, maintaining a pH of 9.5 during the entire procedure, and then clearing in BABB (Schwarz *et al.*, 2015). Matryba *et al.* (2019) reason in their comprehensive review that the

kosmotropic (order-making) nature of tert-butanol might stabilize intramolecular interactions in proteins such as GFP, thus preserving their structure during dehydration.

uDISCO and vDISCO

Ali Ertürk's group reported adaptations of FluoClearBABB for clearing entire animals, in a method they named uDISCO (Pan *et al.*, 2016). Besides good preservation of fluorescent proteins, they also reported significant shrinkage of the cleared organs, which they perceived to be essential for whole-body imaging. The same group also published further optimization of this protocol for whole-body immunohistochemistry, as vDISCO (Cai *et al.*, 2019).

iDISCO and iDISCO+

Whole mount immunolabelling of large specimens was addressed by Renier *et al.* (2016) and Belle *et al.* (2017). Protocols are available online (https://idisco.info/).

A general guidebook for DISCO tissue clearing has recently been published by Ali Ertürk's group (Molbay *et al.*, 2021).

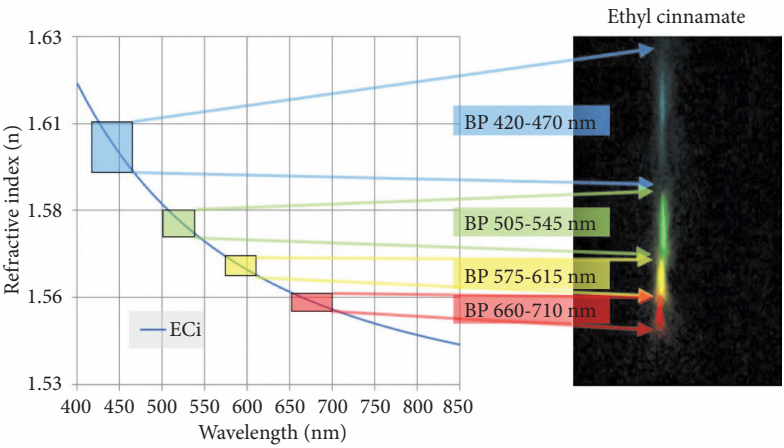
EtCi and 2EtCi

Motivated by "the dramatic loss of signal from fluorescent proteins" and the drawbacks of using "severely toxic substances" such as "benzylalcohol/benzylbenzoate (BABB), dibenzylether (DBE), dichlormethane, or methyl salicylate", Klingberg *et al.* (2017) developed a new protocol for solvent-based clearing to overcome these limitations. This new protocol

utilized the food flavoring ethyl-3-phenylprop-2-enoate (ethyl cinnamate; ECi) as a clearing agent. The authors claim that "ECi is also an excellent clearing reagent for mammalian tissues".

Wouter Masselink, Daniel Reumann and colleagues (2019) describe a "2nd generation ethyl cinnamate based clearing method", which involves combining sample dehydration in propanol at pH 9 followed by refractive index matching with the non-toxic substance ethyl cinnamate. The authors used this protocol "to clear a wide range of tissues, including human organoids, *Drosophila melanogaster*, zebrafish, axolotl and *Xenopus laevis*, in as little as 1–5 days, while preserving a broad range of fluorescent proteins, including GFP, mCherry, Brainbow and Alexa-conjugated fluorophores".

As Daniel Reumann explains (personal communication), researchers around Elly Tanaka's and Jürgen Knoblich's labs, after their breakthrough in growing brain organoids from human stem cells, had been exploring techniques to make those samples transparent. Aqueous-based clearing methods, such as Clarity, did not produce satisfying results, in part due to the low throughput nature of these protocols, but also, the clearing results for organoids were not optimal; presumably due to some tendency of organoids to accumulate necrotic cells. However, organic solvents like BABB were not an option either, because of the toxicity of those reagents and the fact that the same microscopes were used for live imaging. Thus, when the researchers heard about EtCi and the use of pH-matched 1-propanol as a dehydration and delipidation reagent which preserves endogenous fluorescence well, it seemed attractive to combine both. "Two days later I already



had the first cleared organoid with sparse GFP-labelling and it looked great under the microscope", Reumann says.

Ethyl cinnamate is also often used as a non-toxic reagent for mounting samples in light-sheet microscopes, after clearing by other solvent-based protocols such as BABB. The results can be excellent. However, when imaging multiple fluorescent dyes together it should be considered that the refractive index of ethyl cinnamate (n≈1.5-1.6) strongly depends on wavelength. This effect was discussed in detail by Schadwinkel *et al.* (2020).

Summary

Optical clearing of specimens based on removing lipids with organic solvents and refractive index matching is a potent strategy with a long tradition. The clearing effect of these methods is usually robust and reliable. Drawbacks include the reported decay of fluorescence, particularly from fluorescent proteins, as well as the toxicity and corrosiveness of the reagents.

Aqueous-based clearing protocols

Dehydration of histological tissue sections in alcohol, followed by clearing in organic solvents (e.g. xylene) and subsequent mounting in resin (e.g. Euparal), has long been a standard procedure for histochemical applications. Mounting and clearing specimens in aqueous solutions, by contrast, became more popular with the rise of fluorescent stains. This is because fluorescent labels often do not tolerate dehydration, which is necessary for refractive index matching with hydrophobic organic solvents. The rise of 3D optical sectioning techniques, such as confocal laser scanning microscopy (CLSM), and the discovery of genetically encodable fluorescent proteins has further boosted this development since the 1980s.

Aqueous clearing methods have emerged from simple immersion and mounting of specimens (mostly tissue sections) in solutions of compounds that raise the refractive index to the desired value. Such water-soluble optical clearing agents (OCAs) have also been applied to reduce backscattering and increase the transparency of skin for *in vivo* microscopy and other diagnostic and therapeutic approaches.

Sugar

Different mono- and disaccharides have been used as optical clearing agents. First attempts to reduce scattering in biological tissue by glucose can be traced back to Enrico Gratton's lab in 1994. The group reasoned that light scattering in tissue is caused by refractive index mismatches between the aqueous extracellular fluid (ECF) ($n \sim 1.35$) and the lipid cell membranes and proteins ($n \sim 1.35–1.46$). They found that an

increase in ECF glucose levels significantly reduced light scattering and hoped that this parameter could be used for non-invasive blood glucose monitoring (Maier *et al*., 1994).

Feng *et al*. (2016) compared the optical clearing potential of sucrose with that of the monosaccharide fructose on skin and found sucrose to be superior in both depth penetration and fluorescence intensity. The authors also discussed the theoretical background to this observation, and pointed out that glycerol, dimethyl sulfoxide (DMSO), oleic acid, polyethylene glycol, butanediol, sorbitol, xylitol, glucose, fructose, and other compounds had been tested for this purpose before.

Tsai *et al*. (2009) cleared thick mouse brain sections by gradually equilibrating them in increasing concentrations of sucrose in phosphate-buffered saline (PBS), rising to 60%. Without permeabilization, the authors observed significant osmotic shrinkage. For isovolumetric refractive index matching, they added 2% Triton X-100, but a loss of sample integrity was observed for higher detergent concentrations.

Glycerol and DMSO

In 1999, Vargas *et al*. (1999) applied glycerol to reduce light-scattering in skin. In 2006, Moulton *et al*. (2006) found that glycerol and DMSO are effective optical clearing agents on porcine skin for the detection of *Salmonella* infections *in vivo*.

2,2'-Dithioethanol (TDE)

In 2007, the group of Stefan Hell experimented with 2,2'-dithioethanol (TDE) for adjusting the refractive index of

mounting media to values of n = 1.33–1.521. The main motivation for this study was to minimize aberrations in high-resolution microscopy resulting from refractive index mismatches between the sample and the embedding medium (Staudt *et al.*, 2007). Later, TDE was also used for clearing entire mouse brains (Aoyagi *et al.*, 2015) and human brain sections (Costantini *et al.*, 2015), as well as plant tissue (Slane *et al.*, 2017).

Formamide and PEG (CLEAR^{T} and CLEAR^{T2})

Formamide is used for *in situ* hybridization, as a solvent to fine-tune the annealing temperature of the probe. Based on the observation that it also clears tissue samples during the hybridization procedure, Kuwajima *et al.* (2013) developed an optical clearing method, CLEAR^{T}, which relies on immersion in graded concentrations of formamide in PBS. This method has proven successful for clearing intact embryos, embryonic and postnatally dissected heads, brains and thick (up to 1mm) brain sections. It works well for samples labelled with lipophilic tracers such as DiI but failed to preserve fluorescence from heterologous expressed GFP. To stabilize the integrity of GFP, the authors added polyethylene glycol (PEG). The resulting clearing protocol, CLEAR^{T2}, reportedly works well for GFP and immunofluorescence. CLEAR^{T} is, however, faster and leads to better results if GFP and immunofluorescence are not needed. The refractive index of the imaging buffer was n = 1.45 for both methods.

FocusClear™

FocusClear™ is an established, commercialized clearing reagent. According to the manufacturer, it can be applied to a broad variety of samples labelled with fluorescent and non-fluorescent dyes (including lipophilic dyes). Samples can be directly transferred from aqueous solutions, alcohol, DMSO, dimethylformamide (DMF) and glycerine into the FocusClear™ solution and the clearing process should be fast (minutes to hours). The method was first published by Liu and Chiang in 2003, but the authors provided no details on the method or solutions. The refractive index of FocusClear™ is specified as 1.45.

FocusClear™ reagents and the recommended Mount-Clear™ imaging solution are quite costly. The usability of this method therefore remains limited, especially when larger volumes are required (e.g. in light-sheet microscopy). Some researchers have therefore suggested alternative recipes for the imaging solution (e.g. https://forum.claritytechniques.org/discussion/1/FocusClear™).

RIMS

In response to the "prohibitive cost and limited availability" of FocusClear™, Yang *et al.* (2014) described "an affordable substitute": Refractive Index Matching Solution (RIMS). Samples are incubated in this Histodenz™-containing medium until transparent (~ 1–7 days) and then mounted in fresh RIMS. Histodenz™ is a non-ionic density gradient medium used for cell separation.

The refractive index of RIMS is specified as n = 1.46.

Scale

In 2011, Hama *et al*. from Atsushi Miyawaki's team published a clearing method called Sca*l*e that reportedly "renders mouse brain and embryos transparent while completely preserving fluorescent signals from labelled cells". The most effective clearing solution they identified, Sca*l*eA2, contained 4M urea, 10% glycerol and 0.1% Triton X-100. Urea has been shown to non-covalently bind to proteins with a higher affinity than water (Hua *et al*., 2008), thereby breaking up the protein's secondary and tertiary structures, which are primarily responsible for the high refractive index of protein-rich sample domains. Due to this effect on protein structure, Sca*l*eA2 causes significant tissue expansion and the authors therefore developed variants, named Sca*l*eU2 and Sca*l*eB4. Sca*l*eU2 contains 30% glycerol to reduce sample swelling, while Sca*l*eB4 contains 8M urea at pH 8.7, which speeds up the clearing process.

The refractive index of the Sca*l*eA2 imaging solution is around n = 1.38.

ScaleS

In search of ways to further reduce the tissue expansion caused by Sca*l*eA2 and its derivatives, while taking advantage of the fluorescence-preserving features of urea-based clearing protocols, Miyawaki's team discovered that sorbitol improves the procedure (Hama *et al*., 2015). The authors explain that while "urea causes hydration, resulting in tissue expansion, sorbitol causes dehydration, leading to tissue shrinkage". By balancing both effects, they achieved tissue clearing while preserving the original sample volume. Another important

goal of this study was to preserve tissue structure for correlative microscopy. The authors show that brain tissues cleared with Sca*l*eS show a quality and preservation of cellular structures suitable for electron microscopy.

The refractive index of Sca*l*e S is n = 1.44.

SeeDB

Ke *et al*. (2013) developed a fructose-based clearing method for mouse brains, which avoided quenching of fluorescent dyes and allows the use of fluorescent proteins and lipophilic tracers. The method is isovolumetric and takes approximately three days. To avoid browning of the sample due to Maillard reactions, the authors added reducing agents, such as β-mercaptoethanol or α-thioglycerol. A disadvantage of this method is the high viscosity of saturated fructose, which hinders its diffusion into samples larger than mouse brains.

SeeDB2

Ke *et al*. (2016) later described "an optimized optical clearing agent for high-resolution fluorescence imaging (SeeDB2)". They incubated tissue samples in a series of iohexol concentrations in saponin and Tris-EDTA buffer to finally match the refractive indices of the sample to that of immersion oil (n = 1.518). Reportedly, fine morphology and fluorescent proteins were highly preserved during the clearing process.

FRUIT

To circumvent the problem of the high viscosity of saturated fructose solutions making SeeDB unsuitable for sample

perfusion through the vascular system, Hou *et al.* (2015) examined the usability of cocktails of fructose and urea for clearing adult mouse brains. They found that this approach (FRUIT) retains the advantages of SeeDB by preserving fluorescence and lipophilic tracers, but is also compatible with arterial perfusion of larger samples.

CLARITY

As discussed above, sample clearing aims to bring the entire sample to a homogeneous refractive index that is close to the average optical density of all sample components. Unfortunately, water, lipids and proteins have refractive index maxima that are way too far apart from each other to allow effective clearing of all components together. A common strategy is therefore to remove sample components that are less important for the research question.

Aqueous domains ($n \sim 1.3$) can be removed by dehydration, which tends to quench fluorescence. Protein domains ($n \sim 1.6$) could perhaps be digested away enzymatically, but since proteins are usually important for the investigation this is almost never done. Instead, proteins can be hyperhydrated to bring their optical density down closer to that of lipids ($n \sim 1.4$), but this causes significant sample expansion, which can be troublesome, particularly with large samples such as entire brains. Removing lipid domains, such as with sodium dodecyl sulfate (SDS), is an option, but it causes a severe reduction of sample stability and integrity.

In 2013, Chung *et al.* therefore developed a novel technique, CLARITY, for clearing mouse brains, which avoided

significant sample expansion, loss of sample integrity, and highly viscous clearing and imaging solutions. Prior to sample delipidation and refractive index matching, they perfused the animals with paraformaldehyde in phosphate buffer containing hydrogel monomers (e.g. acrylamide and bisacrylamide). After polymerization of the hydrogel, the samples were then incubated and delipidated by 4% SDS in borate buffer; the delipidation was accelerated by electrophoretic removal of the SDS-lipid-micelles. Finally, SDS was removed by excessive washing in PBS. For refractive index matching with the remaining protein domains, the samples were finally incubated in FocusClear™ (n = 1.46).

PACT and PARS

To avoid technical challenges related to the active electrophoretic delipidation of samples in the original CLARITY protocol, and to speed up the time-consuming passive delipidation process, Yang and collaborators in Viviana Gradinaru's team optimized "the hydrogel embedding, clearing, and imaging reagents". The authors claim that the resulting protocol PACT (PAssive CLARITY Technique) allows for "quicker passive lipid extraction of 1–3mm thick tissues" (Yang *et al.*, 2014).

To image PACT-cleared specimens, this group also developed the refractive index matching solution RIMS (n = 1.46), which is described above.

When the authors delivered the PACT reagents by perfusion through the vasculature *in situ* and prior to tissue extraction, they achieved whole body clearing and labelling.

They called this method PARS (perfusion-assisted agent release *in situ*). Reportedly, PARS together with RIMS transformed "opaque, intact, whole-organisms into optically transparent, fluorescently labelled samples".

CUBIC (clear, unobstructed brain/body imaging cocktails)
In 2014, Susaki *et al.* developed a protocol for clearing mouse brains, organs and, finally, entire mouse bodies by modifying and optimizing the urea-based SCALE clearing solutions. The procedure involved immersion in or perfusion by a first reagent containing Triton X-100 and urea for delipidation and (ethylenedinitrilo)tetra-2-propanol (Quadrol) for decolorization, followed by immersion with a second reagent containing triethanolamine, urea and sucrose for refractive index matching to n = 1.49. A variety of solution variants optimized for different sample types are commercially available.

CUBIC (2nd generation)
More recently, Ueda's team embarked on an impressively comprehensive study to optimize their CUBIC protocols (Tainaka *et al.* 2018). The researchers selected 1619 out of 25,000 commercially available chemicals based on their predicted water-solubility and submitted those to a high-throughput evaluation system. The selected chemicals were tested for multiple parameters, including delipidation, decoloring, refractive index, decalcification, and fluorescence quenching of fluorescent proteins.

Based on their findings, the authors suggest a whole set of sample-specific clearing and imaging cocktails, e.g.

CUBIC-L and CUBIC-HL for rapid and fluorescent protein-compatible delipidation and decoloring, CUBIC-RA and CUBIC-R for refractive index matching, CUBIC-P for perfusion clearing of whole animals and CUBIC-B for efficient decalcification. They also provide detailed protocols for adult mouse organs and brain (CUBIC protocol I), mouse whole-bodies including bone (CUBIC protocol II), human tissue (CUBIC protocol III), and large blocks of human brain (CUBIC protocol IV).

In follow-up publications, the group suggested CUBIC-X (Matsumoto *et al.*, 2019) for expansion microscopy and CUBIC-HV as a pipeline for histological analysis on the scale of organs and entire organisms (Susaki *et al.*, 2020).

SWITCH

In 2015, Kwanghun Chung conceived a complex clearing strategy, SWITCH, which aims to improve probe penetration depth and uniformity of staining in large animal and human clinical samples (Murray *et al.*, 2015).

When incubating adult rat brains in 1% glutaraldehyde in PBS at pH 7.4 for two days, Chung and collaborators noted that despite the small size, and thus high mobility, of glutaraldehyde molecules, only the outer layer of the brains were fixed, while the inner parts remained unfixed and totally disintegrated. This is not unexpected, because glutaraldehyde continuously polymerizes in aqueous solution, causing it to lose mobility, and is known to function as a fixative for only a few hours after dilution with a neutral buffer (Kiernan, 2008).

Chung's team took advantage of this effect. When they exposed the tissue to glutaraldehyde for two days at pH 3, and only then switched to pH 7.4, the entire brains were nicely fixed. The authors reason that the glutaraldehyde molecules first homogeneously infiltrate the tissue and only then act as a crosslinker to form a uniform framework throughout the sample, synchronized by the shift in pH. Due to the strong fixation by crosslinking with glutaraldehyde, SWITCH-processed samples endure optical clearing by fast passive lipid removal at 80°C for four days without visible damage.

When the authors added different concentrations of SDS to their buffers, they could also apply the same 'switching' principle to synchronize the binding of antibodies to epitopes throughout the sample. Reportedly, the procedure can be repeated up to 22 times, making this technique a promising tool for diagnostic profiling.

Chung's team also suggested combining SWITCH with SHIELD, a method to preserve fluorescence, immunoreactivity, and nucleic acids in cleared intact tissues by using a polyfunctional, flexible epoxide (Park *et al.*, 2019).

PEGASOS

In 2018, Jing *et al.* published a "general clearing technique applicable for diverse tissues", based on a PEG-associated solvent system (PEGASOS) (Jing *et al.*, 2018). The authors claim that "the PEGASOS method renders nearly all types of tissues transparent except pigmented epithelium. Hard tissues including bones and teeth become nearly invisible after clearing. Polyethylene glycol component within the clearing

medium provided protection for endogenous fluorescence for a long time".

Technically, the method follows a straightforward procedure: 1) fixation by formaldehyde; 2) decalcification by EDTA (for bones); 3) decolorization by Quadrol; 4) delipidation by tert-butanol; 5) dehydration by tert-butanol and PEG; and 6) transfer to a final clearing and imaging solution (n = 1.543), which is composed of 75% benzyl benzoate, 22% methacrylate and 3% Quadrol.

Ce3D

In 2017, Li *et al*. published a clearing method, Ce3D, which they developed to optimize "tissue clarity, conservation of reporter protein fluorescence, optimal preservation of antibody-based staining for multiplex imaging, overall signal quality, morphological integrity at the cell and tissue level, minimal clearing time, and low reagent costs". Systematic testing of various reagents led them to N-methylacetamide in Histodenz for refractive index matching, and for retaining reporter protein fluorescence and the ability to perform multiplex immunolabelling. Thioglycerol and Triton X-100 were added to the final Ce3D clearing solution to minimize colorization and to expedite the clearing process. The authors show impressive clearing of various tissues, including brain, lung, intestine, liver, muscle, thymus and bone. Detailed protocols were published to assist novices with applying the technology (Li *et al*., 2019), which has also been commercialized as a kit.

The viscosity of the final imaging solution is relatively

high, which makes filling sample chambers and moving samples within it a bit challenging. However, the successful use of this method for light-sheet microscopy has recently been published (Ratnayake *et al.*, 2021; Duckworth *et al.*, 2021).

Commercialized clearing methods

An increasing number of tissue-clearing methods are now commercially available. Unfortunately, the details of such procedures often remain undisclosed, which in the author's view contradicts a basic principle of academic research. Some commercial systems are also quite expensive, and cannot easily be modified and optimized for specific purposes due to the lack of information. On the other hand, commercial methods can of course be a pragmatic and reproducible approach for those who want to avoid the effort and time necessary for establishing and optimizing suitable clearing procedures in their lab.

Examples of commercial methods (selected without evaluation and in alphabetical order) include:

- Binaree Tissue Clearing Kit (Binaree, Inc.)
- Ce3D™ (BioLegend)
- CUBIC (Tokyo Chemical Industry)
- CytoVista™ Tissue Clearing Reagent (Invitrogen™)
- FocusClear™ (CelExplorer)
- MACS™ Clearing Kit (Miltenyi Biotech)
- SmartClear (LifeCanvas Technologies)
- Visikol® HISTO™ (Visikol)
- X-Clarity™ (Logos Biosystems).

Clearing of plants

While recent developments in clearing techniques have mostly centered around animal tissues, clearing of plants has commonly been used for decades. In plants, the existence of cell walls and other plant-specific chemical constituents pose additional challenges to microscopic examination, but the handling of samples during the clearing process is often easier due to their higher stability.

Details of classical clearing techniques for plant samples have been nicely described in a review by Gardner (1975). These usually include steps such as fixation, protoplast disruption, decolorization, removal of crystals (e.g. calcium oxalate) and refractive index matching to the cellulose walls (which according to Gardner now form the bulk of the specimens) with clearing agents such as lactic acid, phenol and chloral hydrate.

Chloral hydrate

Chloral hydrate has long been used for clearing plant samples by simple immersion. For references see Villani *et al.* (2013) and the references therein. These authors also compared an acidified chloral hydrate–glycerol solution with the commercially available Visikol clearing agent. They reported that the latter can be effectively used as a replacement for chloral hydrate in botanical microscopy, especially in countries where chloral hydrate is regulated as a narcotic substance with addiction potential.

The refractive indices of chloral hydrate and Visikol range from around 1.44 to 1.46.

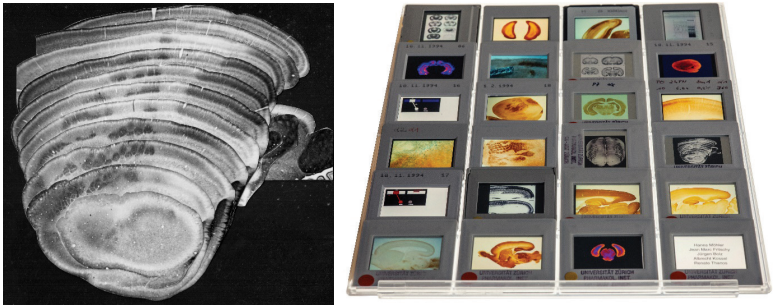
Urea/Glycerol

Warner *et al*. (2014) describe a clearing solution for various leaves and root nodules, based on 6M urea, 30% glycerol and 0.1% Triton X-100 in sterile water. The authors describe their method as "a non-destructive clearing technique, opening unique opportunities for microscope-enabled plant research". Reportedly, their method is compatible with immunocytochemistry and the use of GFP and other fluorescent dyes.

Imaging strategies for large cleared specimens

Physical sectioning of samples has long been the method of choice for obtaining high resolution microscopic images from voluminous specimens and their three-dimensional context. Typically, samples are fixed, cryoprotected (to prevent freezing artifacts) and cut while frozen on a microtome into sections with a thickness of 4–20µm (for light microscopy). These sections are then processed, either attached to gelatin-coated glass slides or free-floating (by applying serial staining solutions with a small brush), and finally mounted under a coverslip for microscopic inspection. This procedure has proven flexible and efficient in research and routine diagnosis, but it is also labour-intensive and requires and ties up skilled labour on a significant scale.

The recent rise of optical tissue-clearing techniques, some of which are described in this ebook, make physical sectioning of samples obsolete in many cases. The previously unattained clarity and transparency of even large samples has allowed optical sectioning technologies to fly to new heights. Several such technologies are commonly used, including structured



Figures: Back in 1994, when the author finalized his first immunohistochemical studies on $GABA_A$-receptor expression in the developing rat brain, staining and clearing techniques were similar to those used today. Three-dimensional analysis, however, was more difficult at that time. Microtome sections of a fixed mouse brain were stained free-floating, after which they were mounted on gelatin-coated glass slides, dehydrated in a series of alcohol concentrations, cleared in xylene and mounted under a coverslip in Euparal. After evaporation of the solvent under a fume hood, the sections were clear, because the Euparal has a refractive index of 1.535; they were then photographed under a microscope. The film was brought to the institute's photo lab, developed, and then printed on paper. To circumvent problems with "the recognition of the overall arrangement", which Spalteholz complained about more than 100 years ago, the sections could then be cut out with a pair of scissors and stacked on top of each other to produce some kind of three-dimensional impression. The rest of the rendering had to be computed in the researcher's mind. Although this approach might sound prehistoric today, the collage could be reproduced on a Kodachrome slide, which was then used for a successful postdoc application talk (Photo: the collage was produced by the author as part of his thesis work in the laboratory of Hanns Mohler and Jean-Marc Fritschy in the Institute of Pharmacology at the University of Zürich, Switzerland).

illumination in Apotome, super resolution-structured illumination microscopy (SR-SIM), confocal laser scanning microscopy (CLSM) and light-sheet fluorescence microscopy (LSFM).

All these technologies are capable of extracting stacks of 2D images from intact 3D samples by identifying and blocking or removing out-of-focus light from the fluorescence images. This is achieved by various methods.

In the Apotome technology, a moving line pattern is projected into the focal plane of a fluorescence microscope

and the out-of-focus signals are identified based on their insensitivity to the movement of the line pattern.[1]

In SR-SIM, optical sectioning is achieved by exciting fluorescence with maximum intensity within the focal plane through interference between the nodes of a light lattice.[2]

Confocal microscopy uses a pinhole in the image plane to block out-of-focus light from entering the photon detector. In the Airyscan technology, an area detector with 32 concentrically arranged detection elements replaces this pinhole. This allows analysis of the photons that are blocked by the pinhole in conventional confocal microscopy, producing higher sensitivity and resolution.[3]

The full potential of optical clearing techniques, however, is realized in light-sheet microscopy. In ZEISS Lightsheet 7, a sample chamber is filled with imaging media, the refractive indices of which can range from n = 1.33 to n = 1.58 due to adjustable optics. A large, cleared sample (e.g. an entire mouse brain) can be glued or otherwise mounted to a sample holder, which moves the sample through a light-sheet in the focal plane of the detection objective. With this approach, optical sectioning is achieved by exciting fluorescence only in the plane of focus, while out-of-focus parts of the sample are left in the dark. This allows gentle imaging at high speed of

[1]https://www.zeiss.com/microscopy/int/products/imaging-systems/apotome-for-biology.html

[2]https://www.zeiss.com/microscopy/int/products/super-resolution/elyra-7.html

[3]https://www.zeiss.com/microscopy/int/products/confocal-microscopes/lsm-980.html

thousands of sections in the z axis and multiple tiles in the x and y axes (if needed) within minutes.[1]

However, when designing the architecture of a light-sheet imaging infrastructure for numerous large, cleared samples, the amount of data generated must be considered. This requires sufficient computing power for rendering and analysis, as well as storage capacity. Concerning the latter, however, which is a frequent concern, one should keep in mind that storage of hundreds of thousands of histological glass slides filling cupboards in the corridors and catacombs of entire institutes has always been a challenge – long before the digital age.

The difference is that today, when using appropriate image analysis and rendering software such as arivis Vision4D[2] or Fiji,[3] hundreds or even thousands of images can be viewed and analyzed simultaneously in their 3D spatial (and for live samples, temporal) context. Optical clearing methods like the ones described in this book, together with computing power, data storage capacity, intelligent rendering and analysis algorithms and, in particular, light-sheet microscopy, now enable analysis of microscopic samples with unprecedented efficiency.

[1]https://www.zeiss.com/microscopy/int/products/imaging-systems/light-sheet-microscope-for-lsfm-imaging-of-live-and-cleared-samples-lightsheet-7.html

[2]https://imaging.arivis.com/en/imaging-science/arivis-vision4d

[3]https://fiji.sc/

Conclusions

More than a century after the birth of tissue optical clearing by Werner Spalteholz, there still is no one-size-fits-all approach for all types of samples. Significant progress has been made for many types of specimens, but every researcher still faces a difficult choice when embarking with new kinds of samples into this rapidly enlarging ocean of clearing technologies.

The good news is that all the available techniques essentially follow the same principles with just minor differences. In general, solvent-based techniques are great because they are inexpensive and reproducible. However, they can shrink tissue significantly, are less likely to be compatible with fluorescent proteins and may wipe out low-expression proteins during the clearing process.

Aqueous techniques such as CUBIC and CLARITY offer better support for fluorescent-protein-expressing tissue, while methods that encase the tissue in a hydrogel or epoxy can improve protein retention. These techniques are thus great for low-expressing targets.

Either way, it is now widely accepted that the final clearing solution should have a refractive index of n = 1.52 or higher.

Case study 1: Going for gold

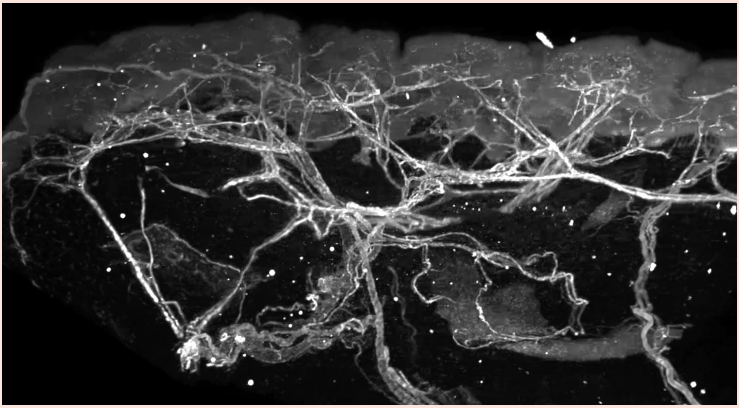
For René Hägerling, research group leader in the Institute of Medical Genetics and Human Genetics at the Charité Universitätsmedizin Berlin, Germany, BABB is the clearing agent of choice.

He and his colleagues use it to clear various human tissues prior to study with light-sheet microscopy. "We use light-sheet technology to perform 3D reconstructions and 3D histological analysis of human tissue biopsies. Our focus was initially on blood and lymphatic vessels in skin tissue, but we've just started using optically cleared tissue samples for studying COVID-19 in lung tissues."

In recent work, they used BABB to clear tissue samples taken from patients with Emberger syndrome, a rare genetic condition caused by the mutation of a single gene that produces a range of symptoms, including tissue swelling in the lower limbs and hearing loss. Hägerling and his colleagues wanted to use light-sheet microscopy to investigate how the swelling alters tissue histology, which previously had only been studied with 2D microscopy techniques (Hägerling, 2020).

"We want to understand the underlying histology and why our patients develop symptoms," Hägerling explains. "For that you need 3D information, because the standard 2D histology is not sufficient to understand complex architecture like the vasculature. Our final aim is to have some kind of decision support based on the histology, for the clinicians treating the patient."

Obtaining this 3D information first required finding the optimum clearing agent for the tissue samples. After testing various different candidates, both organic and aqueous,



Hägerling and his colleagues alighted on BABB as the best option, especially as humans are not naturally fluorescent. "We do not have any endogenously fluorescent units, so we can use organic clearing solutions such as BABB."

Even with BABB, however, getting the tissues sufficiently transparent has proved to be a challenge, requiring Hägerling and his colleagues to modify the standard BABB protocol. This challenge is also greater for some tissues than others. "It depends on tissue size, tissue type, whether it's a dense tissue or a softer tissue."

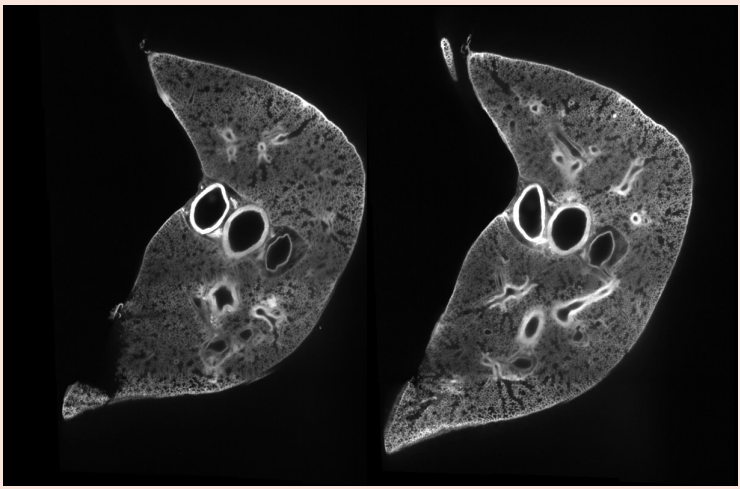
Dark tissues have proved particularly difficult. "BABB could be improved for very dark tissues, such as liver tissue or tissue that is blood filled," Hägerling says. To this end, they've tried combining BABB with bleaching but that has proved trickier than expected.

Despite this, BABB remains their clearing agent of choice, without which they would be unable to probe the 3D structure of human tissue with light-sheet microscopy. "It's our gold standard," Hägerling says.

Case study 2: Clearing makes a difference

As this ebook makes clear, because different biological tissues are made up of different combinations of proteins, lipids and aqueous material, all with different refractive indices, scientists have had to develop a whole range of different clearing agents. That means researchers investigating a variety of different tissues often have to utilize a variety of different clearing agents, as is the case for Alexandre Hego and Sandra Ormenese in the GIGA platform for flow cytometry and cell imaging at the University of Liège in Belgium.

Hego, Ormenese and their colleagues are using light-sheet microscopy to study various different processes in various different tissues. These include the formation of sensory hair cells in the cochlea, the role of immune cells in the lungs, the mechanism of T cell infiltration in cancerous tumors and the dynamics of neurone growth in zebrafish.



To study all these different tissues, they utilize three main types of clearing agent. One is an aqueous solution known as RapiClear, another is a hydrogel produced by combining a version of Clarity known as X-Clarity with RIMS, and the last is CUBIC. Which agent they actually use doesn't just depend on the tissue type, but also on its thickness and the type of staining.

As might be expected, each of these agents has its own combination of advantages and disadvantages. According to Hego, RapiClear has many advantages, including technical simplicity, reversibility and the ability to conserve lipids (allowing lipid staining), but it's expensive. The combination of X-Clarity and RIMS is particularly effective at clarifying the brains of mice with endogenous fluorescence, but it's less effective for other mice organs.

CUBIC is more versatile than X-Clarity and RIMS and less expensive than RapiClear. "We use the CUBIC L-R protocol, which is a revised version of the CUBIC 1-2 protocol," says Hego. "This version allows clarification of all mammalian tissues, unlike the CUBIC 1-2 version, which failed with the heart or lungs of mice."

Indeed, it is this kind of versatility that is allowing Hego, Ormenese and their colleagues to meet all their clearing needs with just these three different agents, rather than requiring many more.

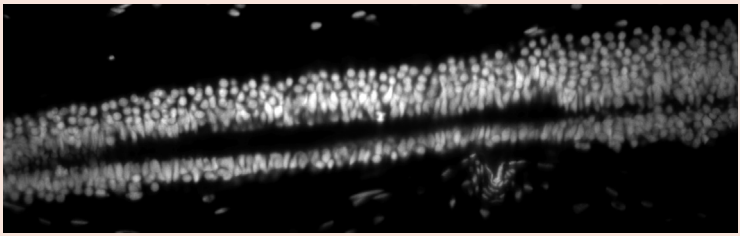
Case study 3: The strange case of the axolotl

*Axolotls (*Ambystoma mexicanum*) are curious animals. Found in several lakes in Mexico and also known as the Mexican walking fish, they are actually a type of salamander, and like many salamanders they have impressive regenerative abilities. Throughout their life, axolotls are able to regenerate and regrow a wide range of complex tissues and structures, including limbs, brain, spinal cord, heart and tail.*

This has inspired many researchers to investigate the mechanisms responsible for these impressive abilities, with the aim of developing novel approaches for regenerating human tissues and organs. One of these researchers is Wilson Pak Kin Lou, a postdoctoral fellow in Elly Tanaka's group at the Research Institute of Molecular Pathology in Vienna, Austria, who is currently using light-sheet microscopy to study neural stem dynamics of spinal cord regeneration in axolotls.

"I do a lot of whole mount stainings to observe structures in 3D space," says Lou, "and clearing is needed for that." And for that clearing, Lou generally turns to ethyl cinnamate (ECi).

Not only has ECi proved very effective at clearing axolotl tissues, but it can also preserve endogenous fluorescence and is compatible with many dye labelling strategies. The Tanaka group



has already used ECi for studies of complex cellular processes and clonal dynamics during axolotl limb regeneration, and now Lou is using it for his studies of spinal cord regeneration.

As with all clearing agents, however, ECi does have certain drawbacks. "Ethyl cinnamate is incompatible with most immersion objectives, and requires a specially designed setup," Lou explains. "Chromatic shift between channels is also more drastic than with other clearing agents." But these drawbacks have not stopped ECi becoming the clearing standard for axolotl tissue in the Tanaka lab.

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Acknowledgements

The author thanks José Rino, Sebastian Munck, Klaus Weisshart, Lorraine Berry, and Abel Pereira da Graca for critical reading of the manuscript; Daniel Reumann, Ali Ertürk, and Andrew Murray for details on the history of their clearing procedures; Douglas Richardson for contributing valuable assessments to the conclusions; and Jon Evans for excellent editorial support.

MICROSCOPY AND ANALYSIS

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