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A survey of clearing techniques for 3D imaging of tissues with special reference to connective tissue

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ABSTRACT

For 3-dimensional (3D) imaging of a tissue, 3 methodological steps are essential and their successful application depends on specific characteristics of the type of tissue. The steps are 1° clearing of the opaque tissue to render it transparent for microscopy, 2° fluorescence labeling of the tissues and 3° 3D imaging. In the past decades, new methodologies were introduced for the clearing steps with their specific advantages and disadvantages. Most clearing techniques have been applied to the central nervous system and other organs that contain relatively low amounts of connective tissue including extracellular matrix. However, tissues that contain large amounts of extracellular matrix such as dermis in skin or gingiva are difficult to clear. The present survey lists methodologies that are available for clearing of tissues for 3D imaging. We report here that the BABB method using a mixture of benzyl alcohol and benzyl benzoate and iDISCO using dibenzylether (DBE) are the most successful methods for clearing connective tissue-rich gingiva and dermis of skin for 3D histochemistry and imaging of fluorescence using light-sheet microscopy.

Contents

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1. Introduction

Anatomical investigations of the human body through surgery and autopsy are the oldest ways to diagnose diseases and to observe effects of therapy. These macroscopical anatomical analyses were already performed in ancient cultures such as those of the Egyptians, Chinese, Persians and Greek. Today, it is still a major approach to deal with diseases such as cancer. However, complementary to macroscopical investigations, microscopical exploration of anatomical structures through histology has been developed in the past centuries since the microscope was invented by Van Leeuwenhoek. Histology has opened a new avenue to understand structure and morphology of tissues and their pathological changes down to the molecular level. It tremendously improved the quality of diagnosis and the monitoring of the effects of therapy in such a way that nowadays histological evaluation is absolutely indispensable. However, traditional 2D histological assessment of tissues limits proper insights in 3D tissue structure. For example, to explore the connectivity of local cellular networks, 3D imaging is essential (Denk and Horstmann, 2004). Imaging of serial sections of a tissue and subsequent 3D reconstruction of the tissue on the basis of those images have been developed successfully (Fig. 1, Griffini et al., 1997), but its application remained limited to small volumes of tissue (Chieco et al., 2013; Chung et al., 2013; Griffini et al., 1997) and it is a time-consuming and error-prone procedure. 3D imaging of tissues is hampered by the fact that tissues are opaque and thus do not allow light to pass what is essential for microscopy. Alternatively, optically-transparent organisms can be used such as embryos of zebrafish (Vittori et al., 2015; Wenner, 2009). However, mammalian tissues including human tissues are not transparent except for the cornea, vitreous, lens and retina in the eye (de Carvalho et al., 2014). Optical tissue clearing (OTC) has been developed to study opaque tissues. As we describe in this review, OTC is performed by immersion of tissues in optical clearing agents to reduce scattering and makes tissues transparent. Tissues are packed with components of different refractive indices (RIs), including scattering particles with high RI such as collagen, elastic fibers, cells, cell membranes and organelles and surrounding media with lower RI i.e. interstitial fluid or cytosol. This architecture forces light to travel at different speeds and angles because each component has different refractive properties (Dodt et al., 2007; Zhu et al., 2010).

Imaging in 3D of tissues has become feasible because of developments in microscopy in the last decades, such as (2-photon) confocal laser scanning microscopy and light sheet microscopy, but the opaqueness of most tissues prevents proper imaging. One of the approaches to facilitate 3D microcopy of tissues is clearing of the tissue.

Recent years have witnessed a burst of high-impact papers in the field of 3D tissue imaging that describe in particular novel methods to clear tissues. In the present review, we discuss which clearing methods are optimal for which conditions.

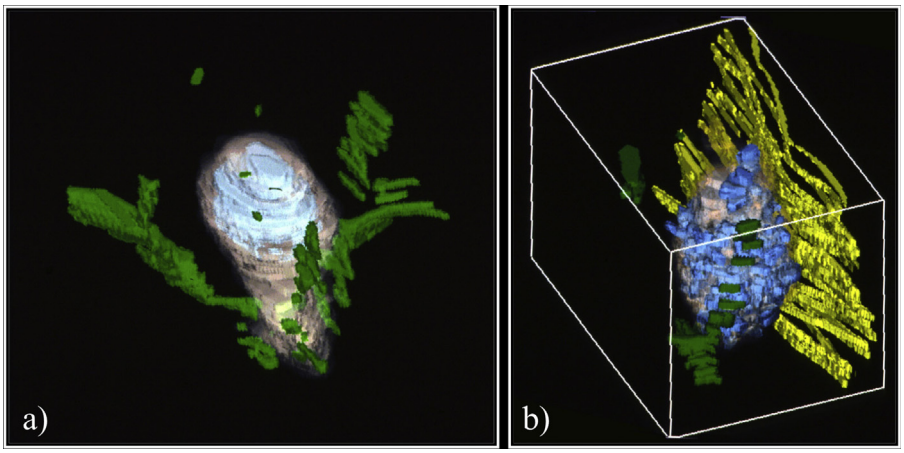

Fig. 1. 3D reconstruction of experimental metastasis of rat colon carcinoma cells in rat liver on the basis of images of 2D serial sections of liver containing the tumors (Griffini et al., 1997). (a) Metastasis of cancer cells (blue) completely encapsulated by connective tissue (pink) with surrounding portal tracts (green). (b) Metastasis showing invasive growth of cancer cells (blue) through surrounding connective tissue (pink). Yellow: lever capsule; green: portal tracts; Bar = 3 mm. Note the artefacts in the reconstruction due to imperfect alignment of the images of serial sections which is particularly evident in the surface of the lever and the portal tracts.

1.1. Clearing methodologies in historical perspective

The German anatomist Werner Spalteholz (1861–1940) (Spalteholz, 1911, 1914) was the first scientist who investigated clearing of tissues. He was studying anastomoses between coronary arteries in the heart and could not make any breakthrough discoveries with standard research methods. Until then (1906), tissues were examined by using preparation techniques for macroscopical and microscopical inspection or by using X-ray diagnostics. Spalteholz realized that for making new discoveries new methods would be necessary and started to investigate alternative methods. Through previous research on muscle tissue, he knew that muscle tissue becomes transparent when dehydrated with the use of alcohol followed by clove oil or xylene and then stored in Canada balsam. He found out that transparency of tissue was highest when light is not reflected on the surface of the tissue, which is the case when the RI of the tissue is the same as the RI of the medium or the solution that holds the tissue. Spalteholz investigated the RI of tissues and found that different tissues have different RIs that also vary with age. He tested different media to match tissue RIs. For example, when benzene was used instead of xylene the RI appeared to be decreased, whereas the RI of carbon disulfide was higher and thus he started to mix solutions. It appeared that when the RI was too high, tissue was not transparent and when the RI was too low, refraction and reflection of light at the surface of the tissue interfered with microscopic visualization. Spalteholz reported a clearing solution that had the ideal chemical characteristics for clearing, namely methyl salicylate and benzyl benzoate (BB) in combination with wintergreen oil. His clearing method was published in 1911 and consisted of the steps that are listed here including modifications introduced since 1911:

1. Preparation of the tissue.
2. Fixation of the tissue using formalin (or better freshly-prepared paraformaldehyde).
3. Decalcification of bone, when present in the tissue samples.
4. Bleaching of the tissue to prevent absorption of light by endogenous pigments. The most simple bleaching procedure is based on the use of hydrogen peroxide to oxidize pigment. The incubation time depends on the consistency of the tissue and the size of the tissue sample. When bleaching with hydrogen peroxide only is not successful due to the tissue consistency, bleaching first using hydrogen peroxide and subsequent fixation using 10% paraformaldehyde may be helpful.
5. Rinsing of tissue using tap water to remove all hydrophilic chemical compounds.
6. Dehydration in an ascending series of alcohol. The final clearing solution is hydrophobic and cannot be mixed with water, thus dehydration is necessary.
7. Incubation in benzene as 100% alcohol can be mixed with benzene. A transfer directly from alcohol to the final clearing solution is possible but alcohol can reduce the transparency of tissue. Therefore, the intermediate step of incubation in benzene improves clearing. Benzene can be removed by using a vacuum pump.
8. Incubation in the clearing solution containing methyl salicylate, BB and wintergreen oil.

Disadvantages of this method were tissue shrinkage due to wintergreen oil and the formation of bubbles due to effects of hydrogen peroxide (Cumley et al., 1939). Necrosis occurred in the outer layers of the tissue (Steinke and Wolff, 2001), which made histological examination difficult.

In the same period of time, Lundvall started similar experiments to clear embryos. He used a mixture of 1 part carbon disulfide and 4 parts benzene, but his results were only satisfying when clearing embryos. The largest drawback of the use of carbon disulfide was, besides its toxicity, the smell that is hard to handle for longer periods of time. Furthermore, he successfully introduced oxalic acid for the bleaching step instead of hydrogen peroxide, which appeared to be more pure but was also more expensive at that time. BB has been frequently used, for example by Orsini in 1962 to show details of reproductive tracts (Orsini, 1962). Her clearing method was based on that of Lundvall, who dehydrated tissues in ascending alcohol series, bleached with hydrogen peroxide and cleared the tissue in BB. However, BB only appeared to be insufficient to achieve complete transparency of tissues, so the method had to be modified further.

Another development of the Spalteholz clearing method was published in 1924 by Ljetnik (1924). He introduced plastics that were generated by polymerization of monomers to replace essential oils such as wintergreen oil and to obtain a block of plastic that contained the tissue.

Eitel et al. (1986) performed macroscopical studies using Spalteholz' preparation technique, and Piechocki (1986) reported new ways to embed tissues. The major problem with these methods was that the treatment of tissues caused tissue damage whereas histological examination was not possible. Peters (1961) demonstrated that a mixture of BB and dimethyl phthalate (DMP) preserved the histology well during the clearing process (Steinke and Wolff, 2001).

von Hagens et al. (1987) developed another method to obtain transparent tissue specimens. His aim was different, because the study was performed to preserve specimens for teaching purposes and as a result he developed a method for plastination of specimens that became famous. It was based on the principle to replace water and lipids in tissues by plastic monomers that then can be polymerized to form plastic blocks containing the tissue sample. There are various ways to plastinate tissues, but tissue slices with a thickness of approx. 2.5 mm are transparent after polymerization.

A combination of the plastination technique of von Hagens et al. (1987) and the clearing technique of Spalteholz (1911) has been investigated as well. Seibold in 1990 tried to combine the methods but failed and Steinke and Wolff (2001) reported that the different RIs of the plastination resin and the clearing solution did not allow clearing of larger specimens. Steinke and Wolff (2001) also reported another modification of the Spalteholz' technique. First, erythrocytes were removed by

rinsing out the blood vessels with a buffer to prevent formation of a brown colour in the tissue samples due to oxidized hemoglobin when cleared with the Spalteholz technique. Furthermore, tissue samples were not chemically fixed using formaldehyde but frozen. However, bleaching was still necessary to prevent that pigments absorb light. Steinke and Wolff bleached with acetone and peracetic acid instead of hydrogen peroxide to minimize structural damage. Then, samples were cleared according to the method of Peters (1961) using BB and DMP. The results of this modified Spalteholz technique were good, especially for histological examination of tissues. Its drawback was that the procedure was time consuming and tissue damage still occurred.

At this point, new developments in microscopy enabled true 3D microscopy of tissues and progress was made rapidly including the clearing methodology. For the study of blood vessels in tissues, various approaches have been used. Steinke and Wolff (2001) obtained the best results by injecting a gelatin solution to keep the lumen of blood vessels open after the gelatin had solidified.

1.2. Clearing methodologies presently used

At present, 10 approaches to clear tissue are used for imaging. They are described in the following chapter. Characteristics of the approaches are summarized in Table 1 .

1.3. BABB

In 2007, Dodt et al. reported that a BABB consisting of 1 part benzyl alcohol (BA) and 2 parts BB was an excellent clearing mixture for mouse brains and embryos and fruit flies (Dodt et al., 2007). This clearing method was applied to clear *Xenopus* embryos and oocytes (Dent et al., 1989; Keller and Dodt, 2012).

BB was introduced as clearing solution by Spalteholz in 1911. Becker et al. (2008) improved the BABB clearing protocol by introducing Dentís Fix solution to prepare mouse embryos and fruit flies for clearing. Dentís Fix contains 1 part dimethylsulfoxide (DMSO) and 4 parts methanol. Bleaching was performed using hydrogen peroxide. Jährling et al. (2009) also cleared mouse brains, spinal cord and hearts using the BABB protocol without using Dent's Fix, while Oldham et al. (2008) also cleared other organs of mice like kidney and liver. However, clearing with the use of BABB was reported to reduce fluorescence signals due to quenching (Yushchenko and Schultz, 2013). This aspect of the BABB clearing method is discussed below. Furthermore, labeling artefacts have been described after BABB clearing (Kuwajima et al., 2013). Furthermore, tissue shrinkage is a problem for clearing procedures based on the use of BABB (Hama et al., 2011; Ke et al., 2013) but also when using other clearing methods. Nevertheless, BABB was one of the first clearing methods developed, it has been used for many different tissues and always resulted in satisfying transparency of tissues.

1.4. Scale

Hama et al. (2011) had observed that polyvinylidene fluoride membranes become transparent in an aqueous solution of urea. This phenomenon inspired the authors to apply urea for clearing of tissues which is an entirely different approach from existing clearing methodologies because urea can be used in aqueous solutions. The major advantage of performing clearing in a hydrophilic environment is that after clearing fluorescence signals in transparent tissues are preserved and enables the application of fluorescently-labeled antibodies against biomarkers (Yushchenko and Schultz, 2013). However, this aspect is discussed below. It is a simple method that does not need specific (expensive) equipment. The RI of urea-containing aqueous solutions is similar to the RI of water, which facilitates imaging with water-immersion objectives. The advantages are the long working distance and the high numeral aperture (Marx, 2014). One of the most effective clearing solutions he tested was Sca*l*eA2, which contains 4 M urea, 10% glycerol and 0.1% Triton X-100. The solution is colorless and is transparent for light with a wavelength >300 nm. Samples maintain their macroscopic structure and quenching of fluorescence signals does not occur, but samples turn soft and fragile due to protein loss (Keller and Dodt, 2012; Steinke and Wolff, 2001). To reduce fragility, Hama et al. (2015) developed the solution Sca*l*eU2, which contains 30% glycerol instead of 10%. Besides a better stability of the tissues, shrinkage of tissue was not observed. Lately Hama et al. (2015) developed another improved method, named Sca*l*eS. The sugar-alcohol sorbitol is combined when using with urea, which results in better preservation of fluorescence and in better transparency than when using Sca*l*eA2.

Hama et al. (2011) tested Sca*l*e only on mouse brain and mouse embryos, but it was assumed that this technique would also be effective when applied to other tissues. Sca*l*eS has been used on postmortem human brain from patients who suffered from Alzheimerís disease (Hama et al., 2015). Becker et al. (2012) critically evaluated clearing using Sca*l*e on mouse brain and muscle preparations. It was concluded that regions in the brain with a high content of myelin and muscle tissue did not become clear at all. Moreover, a drawback is the long incubation time up to months that is needed to render tissue transparent (Keller and Dodt, 2012).

1.5. 3DISCO

Becker et al. (2012) investigated alternatives for the BABB clearing protocol, because it was assumed that BABB clearing does not preserve fluorescence signals. It was found that DBE preserve fluorescence signals better than BABB. DBE has a similar

Table 1
Overview of clearing methods for 3D imaging of tissues and their practical aspect (tissue types tested, duration of the clearing procedure, staining methods tested, protocol of clearing).

Clearing method (non-aqueous clearing)	BABB			3DISCO			iDISCO		
Tissues types tested	Mouse brain and embryo, fruit fly (Dodt et al., 2007); rat heart and lung, mouse brain, tumor and kidney (Oldham et al., 2008)			Mouse mammary gland, lymph node, spinal cord, lung, spleen, brain stem, brain and pancreas (Ertürk et al., 2012a,b, 2014)			Mouse spinal cord, brain, lung and pancreas (Renier et al., 2014)		
Number of steps/duration of procedure	32 steps/4 days			10 steps/4 h-4 days			29 steps/4 days		
(Immuno) histochemical staining tested		Neu-N			FITC			Aquaporin2	
		Steptavidin-Cy3			CD24 α-Smooth muscle actin Alexa Fluor 488			β-Catenin β-Galatosidase cFos Cleaved caspase 3 Cleaved caspase 9 E-cadherin Fox P2 GFP Laminin N-Cadherin Nephrin Neurofilament-M Parvalbumin PeCAM Phospho-cJun Phospho-histone-H3 RFP Ret Robo3 TrkA TrkB TrkC Tuj Tyrosine hydroxylase Cholera toxin β EdU TO-PRO-3	
Protocol	Fix in PFA	Until dehydration	R.T.	Remove blood by perfusion with PFA or PBS			Fix in PFA	Until dehydration	R.T.
	Incubate twice in PBS	Each 1 h		Postfix with PFA	Overnight	R.T.	Incubate twice in PBS	Each 1 h	
	Incubate in 50% methanol in PBS	1 h		Incubate in 50% THF/ 50% PBS	20 min–12 h		Incubate in 50% methanol in PBS	1 h	
	Incubate in 80% methanol in PBS	1 h		Incubate in 70% THF/ 30% PBS	20 min–12 h		Incubate in 80% methanol in PBS	1 h	
	Incubate twice in 100% methanol	Each 1 h		Incubate in 80% THF/ 20% PBS	20 min–12 h		Incubate twice in 100% methanol	Each 1 h	
	Bleach with 5% H_2O_2 in 20% DMSO/methanol (ice cold)	Over-night	4 °C	Incubate in 100% THF	3× 20 min–12 h		Bleach with 5% H_2O_2 in 20% DMSO/methanol (ice cold)	Over-night	4 °C
	Incubate 3 times in 100% methanol	Each 1 h	R.T.	Incubate in DCM	15–45 min		Incubate 3 times in 100% methanol	Each 1 h	R.T.
	Incubate twice in 20% DMSO in methanol	Each 1 h		Incubate in DBE	15 min—1–2 days		Incubate twice in 20% DMSO in methanol	Each 1 h	
	Incubate in 80% methanol in PBS	1 h					Incubate in 80% methanol in PBS	1 h	
	Incubate in 50% methanol in PBS	1 h					Incubate in 50% methanol in PBS	1 h	

Table 1 (*Continued*)

Clearing method (non-aqueous clearing methods)	BABB		3DISCO		iDISCO	
	Incubate twice in PBS	Each 1 h			Incubate twice in PBS	Each 1 h
	Incubate in PBS/0.2% Triton X-100	1 h			Incubate in PBS/0.2% Triton X-100	1 h
	Incubate 4 times in PBS/0.2% Tween-20	Each 1 h			Incubate 4 times in PBS/0.2% Tween-20	Each 1 h
	Incubate in 50% methanol/H_2O	1 h			Incubate in 50% THF/H_2O	Over-night
	Incubate in 70% methanol/H_2O	1 h			Incubate in 80% THF/H_2O	1 h
	Incubate in 80% methanol/H_2O	1 h			Incubate twice in 100% THF	Each 1 h
	Incubate in 96% methanol/H_2O	1 h			Incubate in DCM	Until sample sinks
	Incubate 3 times in 100% methanol	Each 1 h			Incubate in DBE	Until sample is clear
	Incubate in 50% BABB/50% methanol	Over-night				
	Incubate in BABB	Until sample is clear				

Clearing method (aqueous clearing)	Scale		ClearT		SeeDB			CUBIC		
Tissues types tested		Mouse brain and embryo (Hama et al., 2011)		Mouse brain and embryo (Kuwajima et al., 2013)		Mouse brain (Ke et al., 2013)			Mouse brain (Susaki et al., 2014)	
Number of steps/duration of procedure		2 steps/days-weeks (Sca*l*e A2), weeks-months (Sca*l*e U2)		4–6 steps/day		7 steps/3 days			5–7 steps/15–19 days	
(Immuno) histochemical staining tested		FluoroMyelin^{TM} red		RC2		Gephyrin			VIP	
		PSA-NCAM		Mouse monoclonal Neurofilament		Gephyrin (synaptic systems)			Copeptin	
		Brain lipid-binding protein		Immunoglobulin M		Microtubule-associated protein 2			SYTO-16	
		Alexa Fluor 546 Alexa Fluor 633				Alexa 647				
		GLUT1				DAPI				
						Tyrosine hydrolase				
Protocol	Fix in PFA		Fix in PFA or perfuse with PBS at 4 °C		Fix in PFA			Fix in PFA		
	Incubate in Sca*l*eA2 or Sca*l*eU2 until samples are clear	Day–month	Clear^{T}		Incubate in 20% (wt/vol) fructose	4–8 h	25 °C	Incubate in reagent 1	2 × 3 days	37 °C
			Incubate in 40% formamide	5–30 min	Incubate in 40% (wt/vol) fructose			Incubate in PBS several times	–	R.T.
			Incubate in 80% formamide	5 min–2 h	Incubate in 60% (wt/vol) fructose			Incubate reagent 2	3–7 days	37 °C
			Incubate in 95% formamide	5–30 min	Incubate in 80% (wt/vol) fructose	12 h				
			Incubate in 95% formamide until sample is clear	15 min–2 days	Incubate in 100% (wt/vol) fructose					
			Clear^{T2}		Incubate in 80% (wt/vol) fructose	24 h				
			Incubate in 25% formamide/10% PEG	10 min–1 h	Incubate in 87% (wt/vol) fructose		37 °C			
			Incubate in 50% formamide/20% PEG	5 min–1 h						
			Incubate in 50% formamide/20% PEG until sample is clear	15 min–16 h						

Table 1 (*Continued*)

Clearing method (Clarity-based)	Clarity active			Clarity passive			PACT/PARS		
Tissues types tested	Mouse brain, human brain (post mortem) (Chung et al., 2013; Tomer et al., 2014); mouse brain, pancreas, kidney, lung, intestine and liver (Lee et al., 2014); spinal cord (Zhang et al., 2014)			Mouse brain (Chung et al., 2013; Tomer et al., 2014)			Mouse brain, kidney, lung, heart, intestine and tumor (Yang et al., 2014)		
Number of steps/duration of procedure	8 steps/8–22 days			5 + x steps/4–6 weeks up to months				18 steps/32 days	
(Immuno) histochemical staining tested		Alexa Fluor 594 Tyrosine hydrolase *Parvalbumin* Glial fibrillary acidic protein Synapsin-1 Postsynaptic density protein 95 Myelin basic protein Somatostatin Choline acetyltransferase GFP Microtubule-associated protein 2 Neurofilament Taul α-Smooth muscle actin Alexa Fluor 488 DAPI (0.1 μf/mL) NECAB1 NECAB2 CGRP NF200 Calbindin D-28K Calretinin Secretagogin GLUT1 GLUT2 Synaptophysin GFP PKC-γ Cre			Alexa Fluor 594 Tyrosine hydrolase *Parvalbumin* Glial fibrillary acidic protein Synapsin-1 Postsynaptic density protein 95 Myelin basic protein Somatostatin Choline acetyltransferase GFP Microtubule-associated protein 2 Neurofilament			Tyrosine hydrolase Glial fibrillary acidic protein (GFAP) Ionized calcium-binding adaptor molecule 1 Integrin β4, β5 β-Tubulin NeuroTrace 530/615 Red fluorescent Nissl dye	
Protocol	Fix in PFA			Fix in PFA			Fix in PFA by perfusion		
	Embed tissue in AA and BA	2–3 days	4 °C	Embed tissue in AA and BA	2–3 days	4 °C	Perfusion with PBS	2 h	R.T.
	Gas with nitrogen			Gas with nitrogen			Perfusion with 4% acrylamide monomers (A4P0) in PBS	Overnight	
	Polymerize hydrogel	3 h	37 °C	Polymerize hydrogel	3 h	37 °C			
	Remove samples from hydrogel			Remove samples from hydrogel			Perfusion with PBS		
	Incubate in clearing solution	3 × 1 day	37 °C	Incubate in clearing solution until the sample is clear	Change clearing solution every few days	37 °C	Gas with nitrogen gas		
	Incubate in the ETC chamber until the sample is clear	2–16 days	37–50 °C				Polymerization by adding 0.25% VA-044 initiator in PBS		37–42 °C
							Perfusion with 8% SDS buffer in PBS	2 weeks	
							Perfusion with PBS	8× 2 days	

RI as BABB and DBE cleared tissues well. DBE is hydrophobic like BABB, so tissues have to be dehydrated before clearing but because dehydration with a series of ethanol reduces fluorescence, ethanol was replaced by tetrahydrofuran (THF), which had been introduced as clearing agent by Nultsch in 1954. Mouse brains dehydrated in THF and cleared in BABB appeared to be completely transparent. Moreover, THF reduces background fluorescence and thus enhances the specific fluorescence signal. However, tissue shrinkage was observed when using this protocol.

On the basis of the investigations of Becker et al. (2012), Ertürk et al. (2012a) developed a novel clearing method in 2012, called 3DISCO. Clearing was performed on unsectioned brain, spinal cord, mammary glands and lung of mice and it appeared that THF in combination with BABB cleared tissues while fluorescence signals where preserved (Ertürk et al., 2012b). This finding was a breakthrough in THF-based clearing and appeared to be particularly useful for lipid-rich tissues. The first step of the method is dehydration with THF, then tissues are transferred to a dichloromethane (DCM) solution, before clearing is performed in DBE. Tissues became transparent within a few hours, but incubation time in each step depended on the size of the tissue. However, dehydration has to be performed carefully because when dehydration is too fast, more tissue shrinkage is observed, due to the fast removal of water.

3DISCO is compatible with various labeling methods, but tissues cannot be stored in DBE for a longer time, because DBE is aggressive. 3DISCO is now used to clear different types of tissues (Ertürk et al., 2014). Tissue shrinkage is still a problem, but labeled structures remain intact.

1.6. ClearT

Most tissue clearing methods are reported to cause quenching of fluorescence signals and shrinkage of tissues. Kuwajima et al. (2013) introduced an alternative clearing method to solve these problems based on the use of formamide, which is a buffer that is applied in situ hybridisation (Hejatko et al., 2006). The formamide-based clearing method was tested on whole mouse embryos, heads and brains and was named ClearT. Tissues became transparent after incubation in a series of increasing formamide concentrations in buffer, starting with 20% formamide and ending with 95%. The results were comparable to those of other clearing methods. The tissue became as transparent as with Sca*l*e, but the procedure was less time consuming. ClearT also quenched fluorescence but when Kuwajima et al. (2013) added polyethylene glycol (PEG) to the clearing solution to stabilize proteins, it resulted in a novel clearing solution, ClearT2, containing 50% formamide and 20% PEG in buffer. This solution preserves fluorescence signals, but the tissue becomes less transparent than when using ClearT. ClearT also induces tissue volume changes, but they are smaller than when using Sca*l*e or BABB.

1.7. SeeDB

Sca*l*e appeared to be not the optimal aqueous clearing method, because of fragility of samples, and another clearing method was introduced by Ke et al. (2013). The method is called SeeDB (for “see deep brain”) and was developed as aqueous solution, which clears tissues while fluorescent proteins are preserved and not quenched as has been reported to occur when using organic solvents as clearing solvents. Ohtsuki et al. (2012) and Tsai et al. (2009) had described a clearing solution containing high concentrations of sucrose. Ke et al. (2013) investigated various sugar solutions to clear mouse embryos and brains and found a fructose-containing solution to be the ideal solution for clearing purposes. Fructose is highly water soluble and do not cause shrinkage of tissue. Brain samples were cleared in 3 days without any shrinkage or swelling of tissues. It was concluded that tissue swelling as occurs with the Sca*l*e approach was circumvented (Susaki et al., 2014). Furthermore, SeeDB did not cause morphological or chemical changes of tissues. Occasionally, samples turned brownish, but addition of thiols to the clearing solution prevented that. SeeDB is easy to apply, inexpensive and no special equipment is necessary. However, only tissue slices or small tissue specimens can be cleared successfully with SeeDB. Imaging is possible only up to a depth of 1 mm into the tissue (Marx, 2014). Moreover, SeeDB as clearing solution for long-term imaging evaporates. The cleared samples are transparent, but not permeable for macromolecules or antibodies, thus limiting the study of biomarkers in the tissues (Poguzhelskaya et al., 2014).

Calve et al. (2015) also used SeeDB to clear different tissues recently, focusing on skeletal muscle. The authors visualized the morphology of cleared bone and blood vessels in the skull.

1.8. CLARITY

In 2013, Chung et al. presented a completely new method to clear mouse brains, named CLARITY (Chung et al., 2013). CLARITY is based on the concept that lipids have to be removed to clear tissue. However, when lipid bilayers of cell membranes are removed to clear the tissue and to improve diffusion into the tissue, the scaffold of the cell membranes have to be replaced by another framework to stabilize the tissue. For that purpose, tissue was incubated in a solution of acrylamide and bisacrylamide monomers that formed polyacrylamide in the tissue after polymerization (Van Noorden and Tas, 1980; Van Noorden et al., 1984). Then, lipids were removed, using electrophoretic tissue clearing (ETC) instead of clearing using organic solvents because ETC preserved fluorescence. The chamber in which the ETC takes place functions as an electric field, in which the clearing solution circulates. The clearing solution contains boric acid, sodium dodecyl sulfate, sodium hydroxide and distilled water. The ETC chamber is not essential to remove lipids, but when ETC is not used, it takes a long

time to clear tissues and this approach is called passive CLARITY whereas CLARITY with ETC is called active CLARITY. Active CLARITY also takes quite some time to clear tissues completely (Table 1).

CLARITY has been shown to be successful to clear mouse brain and other organs like pancreas, lung or liver (Lee et al., 2014). The time needed for clearing an adult mouse brain is approximately 8 days. In comparison with other clearing methods, CLARITY causes 8% protein loss from tissues during clearing, whereas the protein loss during Sca*l*e clearing, for example, was 41%.

Like all other methods, active CLARITY has disadvantages. For the clearing procedure, specific and expensive equipment is needed and clearing is slow (Kim et al., 2013). The advantage is that CLARITY allows immunostaining afterwards (Tomer et al., 2014). The hydrogel is nanoporous and antibodies can penetrate into the cleared tissues (Poguzhelskaya et al., 2014). Because clearing in the ETC chamber is technically difficult and time consuming, Poguzhelskaya et al. (2014) followed another route with the technique named CLARITY2, also to clear mouse brain. After polymerization and fixation of the hydrogel, tissue samples were sectioned into thick slices to speed up the clearing procedure. This modified CLARITY2 method can be used when thick tissue slices give sufficient information which depends on the purpose of the study.

1.9. CUBIC

Susaki et al. (2014) developed another method to clear mouse brain named CUBIC (clear, unobstructed brain imaging cocktail) that is in fact a modification of Sca*l*e and consists of the subsequent use of two reagents. The first reagent consists of *N*,*N*,*N*′,*N*′-tetrakis(2-hydroxypropyl)ethylenediamine, urea and Triton X-100. Triton X-100 is used to improve diffusion into the tissue (Tsai et al., 2009). The second reagent consisting of sucrose, urea, 2,2′,2′-nitrilotriethanol and Triton X-100 is needed, because the first reagent does not remove all lipids in the tissue specimen. Initially, the concentration of sucrose is 20%, than the tissue is incubated in sucrose solutions with increasingly higher concentrations of sucrose, up to 50%. The first reagent induces some tissue swelling, but the second reagent enables the tissue to return to its normal size. In between the 2 incubations in the clearing reagents, immunostaining can be performed. Fluorescence imaging was possible up to a depth of >750 μm, mainly because permeability of tissues increased after lipid removal.

CUBIC is a simple clearing method and special equipment is not required. It contains only nontoxic water-soluble chemicals, so it is easy to handle. Clearing lasts approximately 2 weeks. The method has only been tested so far on brain tissue.

1.10. PACT/PARS

Another approach to clear tissues was presented by Yang et al. (2014). Mouse brain, kidney, heart and lung have been cleared, as well as human tissue biopsies of basal cell carcinoma. PACT (passive CLARITY technique) and PARS (perfusion-assisted agent release in situ) are both improvements of CLARITY passive clearing methods that take a long time. Yang et al. (2014) tried to find a way to speed up passive clearing and investigated whether decreased cross link density of the hydrogel that embeds the tissue would facilitate lipid extraction. Various concentrations of formaldehyde and acrylamide were used whereas bisacrylamide was omitted. The best results were achieved with 4% acrylamide in buffer which preserved the morphology well. Moreover, step was included to remove oxygen by gassing with nitrogen to speed up polymerization. Instead of the ETC chamber (Chung et al., 2013), 8% sodium dodecyl sulfate was used to extract lipids. This clearing method was called PACT.

PARS is another passive clearing method. The vascular network is used to infuse hydrogel monomers before polymerization and the clearing solution after polymerization. It appeared that tissues were cleared faster and tissue swelling was reduced when using PARS.

1.11. iDISCO

Renier et al. (2014) used mouse brain, embryo, muscle, kidney and spinal cord to develop an improvement of the 3DISCO clearing method that is efficient, rapid, does not require special equipment and can be combined with immunolabeling. The method was a combination of whole-mount immunolabeling using Alexa Fluor dyes and 3DISCO, which was named iDISCO because of a better removal of lipids. Rounds of dehydration and rehydration were included and tissues were bleached as Spalteholz already did in 1914 (Spalteholz, 1914) using hydrogen peroxide and methanol. Bleaching was introduced again to reduce auto-fluorescence of tissues and in combination with methanol it improved immunolabeling. One more pretreatment step was added to the protocol, using DMSO and Triton X-100 to improve penetration of the tissue.

iDISCO is a simple method that results in transparency of various organs, but its disadvantage is that it quenches the fluorescence signal.

1.12. ACT-PRESTO

ACT-PRESTO is the most recently developed clearing method by Lee et al. (2016). It is an abbreviation for: active clearing technique—pressure related efficient and stable transfer of macromolecules into organs.

It was developed for clearing large specimens in an acceptable period of time, which is the great advantage of this new method. While clearing with other techniques takes at least a week, clearing with ACT-PREST can be performed within a day. Furthermore, it is compatible with most immunolabeling techniques and after expanding, the tissue always returns to its original size.

First, the tissue is fixed in a similar way as in the CLARITY protocol (Chung et al., 2013). The main difference is the higher porosity of the resulting hydrogel through acrylamide infusion without bis-acrylamide, which enables a better extraction of lipids. Also the ETC chamber for clearing is modified with a platinum plate and a cooling system. After clearing, the tissue is immunolabeled and washed in PBS before imaging.

Lee et al. (2016) not only cleared various organs of mice, like lung, kidney, thymus, liver and brain, but they also cleared whole bodies of mice, zebrafish and rat embryos successfully.

2. Discussion

The development of confocal microscopy opened the avenue to image cells in 3D with high spatial resolution. Reconstructions of cells in 3D on the basis of stacks of optical sections produced a wealth of information how cells function. Furthermore, fluorescent marker molecules and particularly fluorescent proteins in combination with confocal microscopy promoted our understanding of cell functioning tremendously. Especially, live cell cytochemistry and imaging opened the eyes of cell biologists for the enormous dynamics of cell activity. Cell biological text books had to be rewritten.

The next step is imaging of cells in their natural habitat in tissues. Histologists and histochemists realized that 3D imaging of living tissues will become, no doubt, a similar revolution in our understanding of how tissues function in health and disease as live cell imaging was for the cell biological discipline. Tissues are still studied in 2D in tissue sections and this approach forms the basis of histology text books. Once we are able to perform histology of live tissues in 3D, we will have to rewrite the histology textbooks as well. However, imaging of live tissues in 3D is still a bridge too far. The main reason for that is the opaqueness of tissues that limits imaging to a depth of 500–1000 μm at best (Weigelin et al., 2016) despite the rapid developments in imaging such as 2-photon confocal microscopy.

At present, two major directions are taken to solve these issues. First, imaging of live tissues is performed in the superficial cell layers of a tissue in time to visualize the dynamics in these cell layers (Weigelin et al., 2016) or transparent organisms are used such as zebrafish embryos (Vittori et al., 2015). Second, opaque tissues are being cleared for imaging of a tissue over its entire depth. The present review discusses the clearing methods that are presently available (Table 1). Spalteholz introduced clearing of tissues already a century ago (Spalteholz, 1911), but only in the last decade clearing of tissues was the topic of intense research. The availability and continuous improvements of imaging techniques such as 1-photon and 2-photon microscopy and light-sheet microscopy were a strong stimulus of this research in clearing techniques. The clearing methods that are available now can be roughly divided into 3 groups: First, the CLARITY-based clearing methods such as active CLARITY, passive CLARITY, PACT and PARS, second, the aqueous clearing methods such as SeeDB, CUBIC, ClearT, and Sca*l*e, and third, the non-aqueous clearing methods such as BABB, 3DISCO and iDISCO.

The principle of CLARITY-based methods is the formation of an artificial skeleton of polymerized acrylamide (polyacrylamide). The polyacrylamide skeleton has been introduced in the eighties of last century for quantitative cytochemistry of individual cells and in particular for the visualization and quantification of the activity of enzymes in cells using enzyme cytochemistry, also called metabolic mapping (Fig. 2; De Schepper et al., 1985; Van Noorden et al., 1982, 1984). The artificial polyacrylamide skeleton kept enzymes in their subcellular location and the colored product that was generated by the enzyme under study was precisely localized at the location of the activity of the enzyme (Van Noorden, 2010). This methodology was applied by Chung et al. (2013) for 3D histochemistry and imaging of entire mouse brains. After infusion of acrylamide and bisacrylamide monomers into the brain tissue and subsequent polymerization to build the artificial skeleton, the brain is cleared from lipids to remove cell membranes which clears the brain tissue excellently. However, other types of tissues and in particular extracellular matrix-rich tissues such as connective tissue or stroma (e.g. the dermis in skin or gingiva) cannot be properly cleared by CLARITY-based methods. Brain virtually lacks extracellular matrix and thus opaqueness of the tissue is mainly caused by cell membranes, whereas the extracellular matrix also causes opaqueness of tissue.

The aqueous clearing methods have thus far only been used for tissues without much extracellular matrix such as brain, heart, pancreas and kidney. Furthermore, embryos have been cleared successfully with these aqueous clearing methods but again, mainly for clearing the brain. The images of embryos in these articles all show areas outside the brain where clearing was not successful. Moreover, Renier et al. (2014) reported that resulting transparency is not sufficient for high resolution imaging. Furthermore, Susaki et al. (2014) acquired highly-transparent tissues using 3DISCO and CLARITY, whereas tissues cleared with Sca*l*e and SeeDB did not become transparent. It was proposed that CUBIC is a good alternative to fully clear tissue with an aqueous solution, which should be tested on mouse skin or gingiva in the future. Aqueous solutions change the composition of tissues, which results in a higher permeability. This fact is useful for immunostaining, because penetration of antibodies or dyes is faster (Susaki et al., 2014). According to Genina et al. (2010), Hama et al. (2011) and Ke et al. (2013), another advantage of aqueous clearing solutions is preservation of fluorescence in tissues which was reported not to be the case in hydrophobic clearing solutions such as BABB and DBE. However, fluorescence signals remained intact in our experiments on skin and gingiva clearing with BABB or iDISCO (unpublished data).

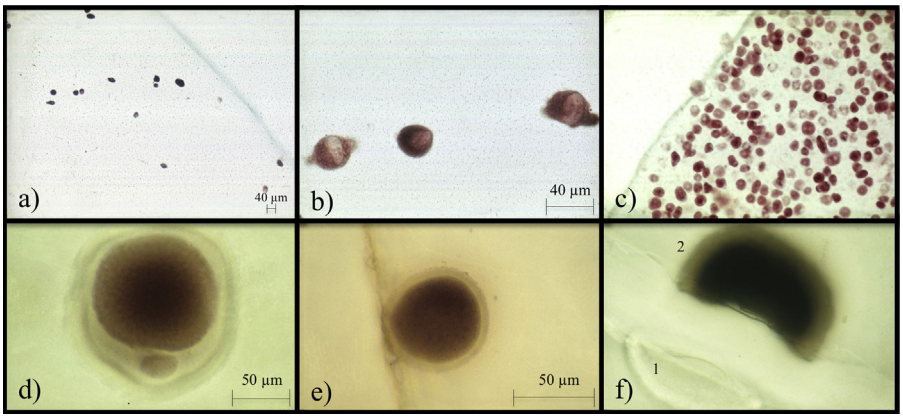

Fig. 2. (a) Isolated fibroblasts incorporated in polyacrylamide gel and then stained for G6PD activity. (b) Larger magnification of fibroblasts incorporated in polyacrylamide and then stained for G6PD activity. c) Isolated hepatocytes incorporated in polyacrylamide and then stained for G6PD activity. (d) Oocyte with polar body incorporated in polyacrylamide gel and then stained for G6PD activity; (e) Oocyte without polar body in polyacrylamide gel after staining for G6PD activity. (f) Oocyte incorporated in polyacrylamide gel that was dissected in two parts and (1) incubated for G6PD activity in the absence of substrate (control) and (2) in the presence of substrate (test). Brownish-reddish colour represents G6PD activity and in (f) the specificity of the staining reaction for G6PD activity is shown because in the presence of substrate colour is generated but not in the absence of substrate.

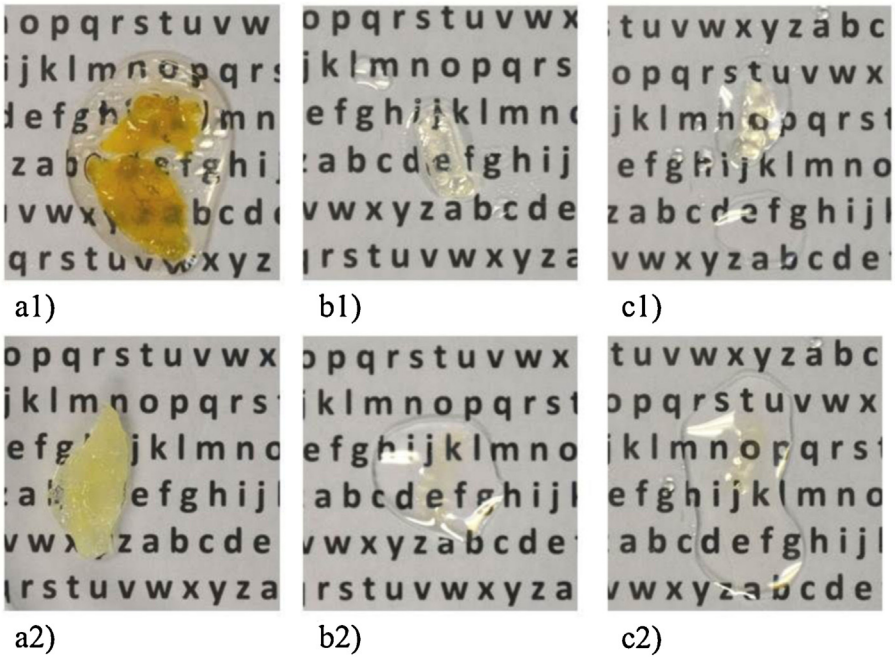

Fig. 3. Clearing of mouse skin and subsequent imaging on lettering paper (type font: Calibri (bold), type size: 8). (a1) Mouse skin cleared with active CLARITY after 4 days of electrophoretic tissue clearing. (a2) Mouse skin cleared with passive CLARITY after 11 weeks of incubation in the clearing solution. (b1) Mouse skin cleared with iDISCO. (b2) Mouse skin cleared with iDISCO and immersed in dibenzylether (DBE) for 15 min. (c1) Mouse skin cleared with BABB. (c2) Mouse skin cleared and immersed in BABB for 30 min.

CUBIC should be preferred to SeeDB and Sca*l*e, because Hama et al. (2011), Ke et al. (2013), Parra et al. (2012) and Poguzhelskaya et al. (2014) describe that samples cleared with Sca*l*e become soft and fragile while samples cleared with SeeDB suffer from limited permeability, and large tissue samples cannot be cleared properly. Alternatively, clearing tissue with SeeDB can be performed at higher temperatures to increase permeability. However, clearing at higher temperatures quenches fluorescence and thus is the advantage of preserved fluorescence lost in samples cleared with aqueous solutions.

The non-aqueous clearing methods such as BABB, iDISCO and probably 3Disco have been excellent tools in our hands to completely clear skin and gingiva (Figs. 3 and 4). Moreover, the clearing itself is ready in a few hours and the entire procedure lasts only 4 h.

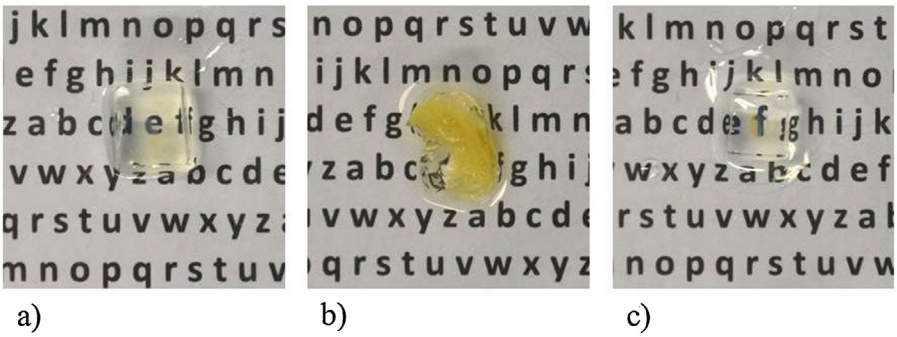

Fig. 4. Clearing of gingiva and subsequent imaging on lettering paper (type font: Calibri (bold), type size: 8). (a) Gingiva embedded in agarose after clearing with BABB. (b) Gingiva after clearing with active CLARITY. (c) Gingiva embedded in agarose after clearing with iDISCO.

Mouse skin and human gingiva did not become optically transparent following active and passive CLARITY clearing, despite the fact that Chung et al. (2013) claim that their method can clear every tissue. Apparently, CLARITY is not suitable for clearing of tissues that contain high amounts of extracellular matrix such as skin or gingiva (Fig. 3a). Furthermore, active CLARITY is a complex method and expensive equipment is needed. These disadvantages of active CLARITY were also reported by Ke et al. (2013), Susaki et al. (2014) and Yang et al. (2014). Passive CLARITY clearing, that does not involve the expensive equipment, may need months to clear a tissue, which does not make it user-friendly.

BABB and DBE-based iDISCO are favorable solutions for connective tissue, because these solutions are easy to use, no special equipment is needed and the clearing procedure is fast. After clearing skin or gingiva according to BABB or iDISCO protocols, all samples became completely transparent (Figs. 3b and 4). Ertürk et al. (2012a) described problems when clearing brain tissue with DBE, but in our study clearing skin and gingiva with iDISCO was fine. The effects of clearing procedures differ for the various tissues (Becker et al., 2012), and skin and gingiva are cleared more effectively with DBE than brain with DBE.

No other clearing methods were tested by us because BABB and iDISCO both cleared skin and gingiva completely in a fast and user-friendly fashion. Clearing with aqueous methods was not tested yet.

A further advantage of BABB and iDISCO is the expedition of the clearing procedures. For example, samples need around two weeks to become transparent when using CUBIC (Susaki et al., 2014). Time for fixing and staining tissue has to be added on top of that. When compared to other aqueous clearing techniques, CUBIC can be considered to be a fast procedure, because other aqueous methods like Sca*l*e need up to six month for proper clearing (Parra et al., 2012). Ertürk et al. (2012a) reported that tissue samples are cleared with 3DISCO within a few hours. Clearing with BABB or iDISCO was even faster. We needed 2.5 days to clear a skin or gingiva sample completely with both methods. Becker et al. (2012) reported that clearing with iDISCO was even faster than with BABB. Becker et al. (2008) needed two days to clear mouse embryos in BABB. However, clearing of skin or gingiva samples lasted only 1–4 h and the entire procedure from collecting tissue samples up to complete transparency lasted 4 days.

Most clearing methods have particular advantages. For example, CLARITY and CUBIC are good for imaging endogenous fluorescence (Renier et al., 2014), Sca*l*e and SeeDB are specifically useful for embryonic tissue (Ertürk et al., 2014). Ertürk et al. (2012a) describe 3DISCO to be useful for clearing of all types of tissues. We like to add here that BABB and iDISCO are useful for extracellular matrix-rich tissues such as skin and gingiva.

When comparing transparency of samples cleared with BABB and iDISCO, no differences could be observed in the present study (Figs. 3 and 4). This contradicts the results of Ertürk et al. (2012a) and Becker et al. (2012), who found samples to be cleared with DBE to be more transparent than those cleared with BABB.

Protocols describing clearing with BABB vary, such as those of Becker et al. (2008, 2013, 2012), Jährling et al. (2009) and Parra et al. (2012). The main difference between the protocols is the final dehydration step, which can be performed with the use of either ethanol or THF. The present study is based on the protocol of Becker et al. (2012), using THF. Ertürk et al. (2012a,b) and Becker et al. (2012) state that THF better preserves fluorescence signals. In our experiments fluorescence signals were preserved as well. We also used autofluorescence signals of gingiva for imaging. Therefore, it can be concluded that BABB and iDISCO containing DBE are methods that preserve fluorescence.

Whether fluorescence is quenched or not in hydrophobic clearing solutions such as BABB and DBE appears to be not investigated very well. When studying the relevant literature (Becker et al., 2012; Ertürk et al., 2012a,b; Genina et al., 2010; Hama et al., 2011; Ke et al., 2013; Kuwajima et al., 2013; Yushchenko and Schultz, 2013), it appears that a proper analysis of the effects of a clearing solution on fluorescence signals has not been performed. Moreover, the literature on effects of BABB or DBE on fluorescence signals is not equivocal (Becker et al., 2012; Ertürk et al., 2012a,b; Yushchenko and Schultz, 2013) and experts in the fluorescence field that we have consulted are convinced that fluorescence is not dependent on an aqueous environment and assume that it depends on the fluorescent molecule whether it fluoresces in an hydrophobic environment as we have observed ourselves with Alexa dyes, phycoerythrin and autofluorescence (unpublished data). It may be possible

that fluorescent proteins are quenched in hydrophobic clearing solutions because the 3D molecular structure of proteins is lost, but again, this has to be investigated properly.

To reduce background signals while imaging, Renier et al. (2014) recommended to treat samples with heparin before the final dehydration. However, this treatment appeared to have quenching effects. As methanol has also quenching effects, Renier et al. (2014) suggested a modified clearing method without using methanol. The additional step of heparin treatment makes the clearing process more time consuming. In the present study, loss of fluorescence signals was not observed and thus, amendments of the iDISCO protocol as suggested by Renier et al. (2014) were not tested.

Although it has been reported that BABB and DBE quench fluorescence signals in tissues (Ertürk et al., 2012a), it was also found that even after 3 months of storage, fluorescence signals were still be detectable in tissues stored in BABB. Alexa Fluor® fluorescence was found to be stable for a few months in BABB (Renier et al., 2014).

Besides, differences in the effect of dehydration with ethanol or methanol have also been described. Parra et al. (2012) proposed ethanol to be used for better preservation of fluorescence signals. Becker et al. (2008) and Jährling et al. (2009) corroborate these findings and use ethanol for dehydration. In our study, methanol was used for clearing of gingiva samples and we did not find any loss of fluorescence signals, irrespective whether the iDISCO or BABB protocol was followed.

Clearing with organic solvents has the disadvantage of toxicity and aggressiveness. Organic clearing solutions have to be handled carefully. Parra et al. (2012) found out that BABB can dissolve glue. Therefore, application of BABB and DBE in Petri dishes and coverslips was tested first before imaging gingiva. Our findings were in agreement with those of Parra et al. (2012), as BABB and DBE indeed dissolved glue. As an alternative, dental cement was tried for imaging (Ertürk et al., 2012a). The ring formed on a coverslip did not dissolve, but did not stick properly to the glass either. Therefore, this approach could not be used. However, a metal ring around the tissue sample solved the problem for confocal microscopy whereas light-sheet microscopes have a chamber especially designed for solutions like DBE and BABB.

Imaging is only successful when the appropriate imaging solution is used. When the RI of the tissue is different from that of the imaging solution, light is scattered which blurs the image and limits the penetration of the laser light into the tissue sample. Glycerol cannot be used as imaging solution because according to Richardson and Lichtman (2015) the RI of the imaging solution and the tissue and glass coverslips and immersion oil differs too much. Moreover, glycerol is viscous, and bubbles are introduced easily during handling, which makes imaging impossible. Finally, glycerol is hydrophilic, while BABB and DBE are hydrophobic, and glycerol does not penetrate the tissue that has been cleared with DBE (Ertürk et al., 2014). The best results were obtained when imaging the tissue in its clearing solution. Tissues that were cleared in BABB can also be imaged in DBE as imaging solution, because the RIs of both solutions are nearly identical. For tissue cleared in aqueous solutions, other imaging solutions than glycerol have been proposed. FocusClear has been used by Chung et al. (2013) to image tissue that has been cleared with CLARITY. It contains DMSO, diatrizoate acid and other reagents (Genina et al., 2010), but the exact composition is proprietary. As a result, FocusClear is expensive and cannot be optimized for the various tissues other than brain (Hama et al., 2011). Although it has been described for imaging tissue using a confocal microscope (Chiang et al., 2001; Chung et al., 2013; Fu et al., 2009), it was not necessary to test imaging with FocusClear in the present study. Marx (2014) also reported that FocusClear is not necessary.

To summarize our review of methods particularly for clearing human extracellular-rich tissue, it can be stated, that the best results are obtained when these tissues are cleared with BABB or iDISCO.

Conflict of interest

The authors declare that they have no conflict of interests.

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References

Becker, K., Jahrling, N., Kramer, E.R., Schnorrer, F., Dodt, H.U., 2008. Ultramicroscopy: 3D reconstruction of large microscopical specimens. J. Biophotonics 1 (1), 36–42.

Becker, K., Jahrling, N., Saghafi, S., Dodt, H.U., 2013. Immunostaining, dehydration, and clearing of mouse embryos for ultramicroscopy. Cold Spring Harb. Protoc. 2013 (8), 743–744.

Becker, K., Jahrling, N., Saghafi, S., Weiler, R., Dodt, H.U., 2012. Chemical clearing and dehydration of GFP expressing mouse brains. PLoS One 7 (3), e33916.

Calve, S., Ready, A., Huppenbauer, C., Main, R., Neu, C.P., 2015. Optical clearing in dense connective tissues to visualize cellular connectivity in situ. PLoS One 10 (1), e0116662.

Chiang, A.-S., Liu, Y.-C., Chiu, S.-L., Hu, S.-H., Huang, C.-Y., Hsieh, C.-H., 2001. Three-dimensional mapping of brain neuropils in the cockroach, *Diploptera punctata*. J. Comp. Neurol. 440 (1), 1–11.

Chieco, P., Jonker, A., De Boer, B.A., Ruijter, J.M., Van Noorden, C.J.F., 2013. Image cytometry: protocols for 2D and 3D quantification in microscopic images. Prog. Histochem. Cytochem. 47 (4), 211–333.

Chung, K., Wallace, J., Kim, S.Y., Kalyanasundaram, S., Andalman, A.S., Davidson, T.J., Mirzabekov, J.J., Zalocusky, K.A., Mattis, J., Denisin, A.K., Pak, S., Bernstein, H., Ramakrishnan, C., Grosenick, L., Gradinaru, V., Deisseroth, K., 2013. Structural and molecular interrogation of intact biological systems. Nature 497 (7449), 332–337.

Cumley, R.W., Crow, J.F., Griffen, A.B., 1939. Clearing specimens for the demonstration of bone. Biotech. Histochem. 14 (1), 7–11.
de Carvalho, J.E.R., Verbraak, F.D., Aalders, M.C., van Noorden, C.J., Schlingemann, R.O., 2014. Recent advances in ophthalmic molecular imaging. Surv. Ophthalmol. 59 (4), 393–413.
De Schepper, G., Van Noorden, C., Koperdraad, F., 1985. A cytochemical method for measuring enzyme activity in individual preovulatory mouse oocytes. J. Reprod. Fertil. 74 (2), 709–716.
Denk, W., Horstmann, H., 2004. Serial block-face scanning electron microscopy to reconstruct three-dimensional tissue nanostructure. PLoS Biol. 2 (11), e329.
Dent, J.A., Polson, A.G., Klymkowsky, M.W., 1989. A whole-mount immunocytochemical analysis of the expression of the intermediate filament protein vimentin in *Xenopus*. Development 105 (1), 61–74.
Dodt, H.U., Leischner, U., Schierloh, A., Jahrling, N., Mauch, C.P., Deininger, K., Deussing, J.M., Eder, M., Zieglgansberger, W., Becker, K., 2007. Ultramicroscopy: three-dimensional visualization of neuronal networks in the whole mouse brain. Nat. Methods 4 (4), 331–336.
Eitel, F., Seibold, R., Hohn, B., Schweiberer, L., 1986. Präparationstechnische Weiterentwicklung und Standardisierung der mikroangiographischen Untersuchungsmethode nach Spalteholz. Unfallchirurg 89, 326–336.
Ertürk, A., Becker, K., Jährling, N., Mauch, C.P., Hojer, C.D., Egen, J.G., Hellal, F., Bradke, F., Sheng, M., Dodt, H.-U., 2012a. Three-dimensional imaging of solvent-cleared organs using 3DISCO. Nat. Protoc. 7 (11), 1983–1995.
Ertürk, A., Mauch, C.P., Hellal, F., Forstner, F., Keck, T., Becker, K., Jahrling, N., Steffens, H., Richter, M., Hubener, M., Kramer, E., Kirchhoff, F., Dodt, H.U., Bradke, F., 2012b. Three-dimensional imaging of the unsectioned adult spinal cord to assess axon regeneration and glial responses after injury. Nat. Med. 18 (1), 166–171.
Ertürk, A., Lafkas, D., Chalouni, C., 2014. Imaging cleared intact biological systems at a cellular level by 3DISCO. J. Vis. Exp. 89.
Fu, Y.Y., Lin, C.W., Enikolopov, G., Sibley, E., Chiang, A.S., Tang, S.C., 2009. Microtome-free 3-dimensional confocal imaging method for visualization of mouse intestine with subcellular-level resolution. Gastroenterology 137 (2), 453–465.
Genina, E.A., Bashkatov, A.N., Tuchin, V.V., 2010. Tissue optical immersion clearing. Expert Rev. Med. Devices 7 (6), 825–842.
Griffini, P., Smorenburg, M., Verbeek, F.J., Van Noorden, C.J.F., 1997. Three-dimensional reconstruction of colon carcinoma metastases in liver. J. Microsc. 187 (Pt 1), 12–21.
Hama, H., Hioki, H., Namiki, K., Hoshida, T., Kurokawa, H., Ishidate, F., Kaneko, T., Akagi, T., Saito, T., Saido, T., 2015. Sca*l*eS: an optical clearing palette for biological imaging. Nat. Neurosci. 18 (10), 1518–1529.
Hama, H., Kurokawa, H., Kawano, H., Ando, R., Shimogori, T., Noda, H., Fukami, K., Sakaue-Sawano, A., Miyawaki, A., 2011. Sca*l*e: a chemical approach for fluorescence imaging and reconstruction of transparent mouse brain. Nat. Neurosci. 14 (11), 1481–1488.
Hejatko, J., Blilou, I., Brewer, P.B., Friml, J., Scheres, B., Benkova, E., 2006. In situ hybridization technique for mRNA detection in whole mount *Arabidopsis* samples. Nat. Protoc. 1 (4), 1939–1946.
Jährling, N., Becker, K., Dodt, H.-U., 2009. 3D-reconstruction of blood vessels by ultramicroscopy. Organogenesis 5 (4), 227–230.
Ke, M.T., Fujimoto, S., Imai, T., 2013. SeeDB: a simple and morphology-preserving optical clearing agent for neuronal circuit reconstruction. Nat. Neurosci. 16 (8), 1154–1161.
Keller, P.J., Dodt, H.-U., 2012. Light sheet microscopy of living or cleared specimens. Curr. Opin. Neurobiol. 22 (1), 138–143.
Kim, S.-Y., Chung, K., Deisseroth, K., 2013. Light microscopy mapping of connections in the intact brain. Trends Cogn. Sci. 17 (12), 596–599.
Kuwajima, T., Sitko, A.A., Bhansali, P., Jurgens, C., Guido, W., Mason, C., 2013. Clear(T): a detergent- and solvent-free clearing method for neuronal and non-neuronal tissue. Development (Cambridge, England) 140 (6), 1364–1368.
Lee, E., Choi, J., Jo, Y., Kim, J.Y., Jang, Y.J., Lee, H.M., Kim, S.Y., Lee, H.-J., Cho, K., Jung, N., 2016. ACT-PRESTO: rapid and consistent tissue clearing and labeling method for 3-dimensional (3D) imaging. Sci. Rep. 6.
Lee, H., Park, J.-H., Seo, I., Park, S.-H., Kim, S., 2014. Improved application of the electrophoretic tissue clearing technology, CLARITY, to intact solid organs including brain, pancreas, liver, kidney, lung, and intestine. BMC Dev. Biol. 14 (1), 1.
Ljetnik, S., 1924. Eine variation der spalteholzschen methode. Anat. Anz. 59, 201–202.
Marx, V., 2014. Microscopy: seeing through tissue. Nat. Methods 11 (12), 1209–1214.
Nultsch, W., 1954. Über die Verwendung des Tetrahydrofurans in der mikroskopischen Technik.
Ohtsuki, G., Nishiyama, M., Yoshida, T., Murakami, T., Histed, M., Lois, C., Ohki, K., 2012. Similarity of visual selectivity among clonally related neurons in visual cortex. Neuron 75 (1), 65–72.
Oldham, M., Sakhalkar, H., Oliver, T., Allan Johnson, G., Dewhirst, M., 2008. Optical clearing of unsectioned specimens for three-dimensional imaging via optical transmission and emission tomography. J. Biomed. Opt. 13 (2), 021113.
Orsini, M.W., 1962. Technique of preparation, study and photography of benzyl-benzoate cleared material for embryological studies. J. Reprod. Fertil. 3, 283–287.
Parra, S.G., Vesuna, S.S., Murray, T.A., Levene, M.J., 2012. Multiphoton microscopy of cleared mouse brain expressing YFP. J. Vis. Exp. 67, e3848.
Peters, W., 1961. Methoden zur Herstellung von Aufhellungspräparaten. Zool. Anz. 167, 233–240.
Piechocki, R., 1986. Makroskopische Pr∼ iparationstechnik, Bd.
Poguzhelskaya, E., Artamonov, D., Bolshakova, A., Vlasova, O., Bezprozvanny, I., 2014. Simplified method to perform CLARITY imaging. Mol. Neurodegener. 9, 19.
Renier, N., Wu, Z., Simon, D.J., Yang, J., Ariel, P., Tessier-Lavigne, M., 2014. iDISCO: a simple, rapid method to immunolabel large tissue samples for volume imaging. Cell 159 (4), 896–910.
Richardson, D.S., Lichtman, J.W., 2015. Clarifying tissue clearing. Cell 162 (2), 246–257.
Spalteholz, W., 1911. Über das Durchsichtigmachen von Menchlichen und Tierichen Präparaten und Seine Theoretischen Bedingungen. S. Hirzel, Leipzig.
Spalteholz, W., 1914. Über das Durchsichtigmachen von Menschlichen und Tierischen Präparaten und Seine Theoretischen Bedingungen. S. Hirzel, Leipzig.
Steinke, H., Wolff, W., 2001. A modified Spalteholz technique with preservation of the histology. Ann. Anat. Anat. Anz. 183 (1), 91–95.
Susaki, E.A., Tainaka, K., Perrin, D., Kishino, F., Tawara, T., Watanabe, T.M., Yokoyama, C., Onoe, H., Eguchi, M., Yamaguchi, S., Abe, T., Kiyonari, H., Shimizu, Y., Miyawaki, A., Yokota, H., Ueda, H.R., 2014. Whole-brain imaging with single-cell resolution using chemical cocktails and computational analysis. Cell 157 (3), 726–739.
Tomer, R., Ye, L., Hsueh, B., Deisseroth, K., 2014. Advanced CLARITY for rapid and high-resolution imaging of intact tissues. Nat. Protoc. 9 (7), 1682–1697.
Tsai, P.S., Kaufhold, J.P., Blinder, P., Friedman, B., Drew, P.J., Karten, H.J., Lyden, P.D., Kleinfeld, D., 2009. Correlations of neuronal and microvascular densities in murine cortex revealed by direct counting and colocalization of nuclei and vessels. J. Neurosci. 29 (46), 14553–14570.
Van Noorden, C., Tas, J., 1980. Quantitative aspects of the cytochemical demonstration of glucose-6-phosphate dehydrogenase with tetranitro BT studied in a model system of polyacrylamide films. Histochem. J. 12 (6), 669–685.
Van Noorden, C.J.F., Tas, J., Vogels, I., De Schepper, G., 1982. A new method for the enzyme cytochemical staining of individual cells with the use of a polyacrylamide carrier. Histochemistry 74 (2), 171–181.
Van Noorden, C.J.F., Vogels, I., Houtkooper, J., Fronik, G., Tas, J., James, J., 1984. Glucose-6-phosphate dehydrogenase activity in individual rat hepatocytes of different ploidy classes. I. Developments during postnatal growth. Eur. J. Cell Biol. 33 (1), 157–162.
Van Noorden, C.J.F., 2010. Imaging enzymes at work: metabolic mapping by enzyme histochemistry. J. Histochem. Cytochem. 58 (6), 481–497.
Vittori, M., Motaln, H., Turnšek, T.L., 2015. The study of glioma by xenotransplantation in zebrafish early life stages. J. Histochem. Cytochem. 63 (10), 749–761.
von Hagens, G., Tiedemann, K., Kriz, W., 1987. The current potential of plastination. Anat. Embryol. (Berl.) 175 (4), 411–421.
Weigelin, B., Bakker, G.-J., Friedl, P., 2016. Third harmonic generation microscopy of cells and tissue organization. J. Cell Sci. 129 (2), 245–255.
Wenner, M., 2009. The most transparent research. Nat. Med. 15 (10), 1106–1109.

Yang, B., Treweek, J.B., Kulkarni, R.P., Deverman, B.E., Chen, C.K., Lubeck, E., Shah, S., Cai, L., Gradinaru, V., 2014. Single-cell phenotyping within transparent intact tissue through whole-body clearing. Cell 158 (4), 945–958.
Yushchenko, D.A., Schultz, C., 2013. Geklärte Gewebeproben für die optische Anatomie. Angew. Chem. 125 (42), 11151–11154.
Zhang, M.D., Tortoriello, G., Hsueh, B., Tomer, R., Ye, L., Mitsios, N., Bergius, L., Grant, G., Kiehn, O., Watanabe, M., Uhlen, M., Mulder, J., Deisseroth, K., Harkany, T., Hokfelt, T.G., 2014. Neuronal calcium-binding proteins 1/2 localize to dorsal root ganglia and excitatory spinal neurons and are regulated by nerve injury. Proc. Natl. Acad. Sci. U. S. A. 111 (12), E1149–E1158.
Zhu, D., Wang, J., Zhi, Z., Wen, X., Luo, Q., 2010. Imaging dermal blood flow through the intact rat skin with an optical clearing method. J. Biomed. Opt. 15 (2), 026008.