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REVIEWS

TISSUE CLEARING PROTOCOLS: AN OVERVIEW OF CURRENT METHODS AND APPROACHES

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ABSTRACT

Observation of complex cellular shapes in three-dimensional (3D) tissue context requires the use of thick blocks of tissue samples, which are not transparent. To overcome this optical opacity, a specific set of histological techniques have been developed and named tissue clearing methods. Here we review the major available tissue clearing protocols and justify the advantages of a particular protocol of tissue clearing named CUBIC over the other methods in imaging and 3D reconstruction.

Keywords: *tissue clearing, 3D microscopy, CUBIC protocol*

INTRODUCTION

Three-dimensional (3D) optical visualization of cells in the tissues has recently become widely available. Although it requires the use of specialized microscopes, the modern development in microscopy has lead to the wide availability of such equipment around the globe. Nevertheless, the visualization of complex cellular shapes and processes in the 3D space requires the application of very thick sections or even whole blocks of tissue. The main visualization problem when using thick sections is the light scattering. To overcome this impediment of acquiring images of good resolution, over the last two decades a number of techniques aiming to make the tissues transparent to light have been proposed. They are collectively known as *tissue clearing* techniques (1). Such techniques use a workflow, which includes applying consecutively chemical compounds that remove lipids, carbohydrates and pigments, but leave proteins intact to allow labeling via immunohistochemical techniques.

Most of the tissues in the human body except the cornea, the vitreous body, or the lens are not transparent and light cannot pass freely through them (2). This is where tissue clearing methods come into play. Tissues are impenetrable to light because tissue components such as collagen, elastic fibers, organelles, cytosol, and cell membranes are made up of water, lipids, proteins, and minerals distributed in uneven amounts with different refractive index. The transparency required for the light to pass freely depends on the refractive index of the tissue constituents, which must be gradually equalized in order to avoid the effect of light scattering and absorption (2). Equalization of the refractory index of various substances like lipids and proteins facilitates the

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Table 1. Comparison of major tissue clearing methods. See the text for the full name of each method.

Protocol/	BABB[7]	ScaleS[10]	DISCO[9]	SeeDB[11]	CLARITY[12]	CUBIC[6]
Year	**2007**	**2011**	**2012**	**2013**	**2013**	**2014**
Chemical compounds for delipidation, depigmentation and decolorization	Benzyl alcohol/ benzyl benzoate (BABB) refractive index (RI) match [15] Depigmentation - none [15] Dehydration - ethanol [15]	ScaleA2 (urea, glycerol and Triton X-100) [15] Depigmentation - None Hyperhydration - Urea [16]	Dibenzyl ether (DBE) for RI match [15] Depigmentation H_2O_2 [15] Dehydration Tetrahydrofuran (THF) [15]	Fructose and sucrose for RI match [15] Depigmentation - Thioglycerol [15] Dehydration - Fructose [14]	Passive clearing Sodium dodecyl sulfate (SDS) or 'Electrophoretic tissue clearing' (ETC) based clearing for depigmentation and dehydration [15]	Reagent 2: urea, aminoalcohol and sucrose for RI match [15] Reagent 1: Delipidation -Triton X-100 [15] Hyperhydration - Urea, Sucrose [15]
Advantages	The method is inexpensive [8] Clearing time is rapid (few days) [8]	Preserves fluorescence signals [3] The method is inexpensive [3]	Preserves [3] fluorescence signals Clearing time is rapid (few days)[8] The method is inexpensive [8]	Preserves fluorescent signals [3] The methods is inexpensive [3] Can be used for skeletal muscle and bones [3]	Immunostaining after clearing procedure [8]	Preserves fluorescence signals [3] The method is inexpensive [3]
Disadvantages	- Tissue shrinkage [8] - Reduces fluorescence signals [8] - Uses Toxic compounds [8]	- The sample turns soft and fragile due to protein loss [3] - Tissue expansion is typically not uniform causing alter the structure and morphology [8]	- Tissue shrinkage[3] - Uses Toxic compounds [8]	- Uses only tissue slices or small tissue specimens[3] - Imaging is possible to a depth of 1mm [3]	- Specific and expensive equipment [3] - Clearing time is slow [3]	- Clearing time is very variable (from hours to weeks) [8] - Tissue expansion is typically not uniform causing altered structure and morphology [8]
Main categories: non-aqueous based or aqueous based	Non-aqueous solvent based clearing techniques [8]	Aqueous based clearing techniques - hyperhydratation method [8]	Non-aqueous solvent based clearing techniques [8]	Aqueous based clearing techniques - simple immersion [8]	Non-aqueous hydrogel embedding methods [8]	Aqueous based clearing techniques - hyperhydratation method [8]
Clearing performance	High[14]	Moderate[14]	Highest[14]	Weak[14]	ETC - highest[14]	High[14]

passage of light through the tissue and reduces light scattering.

The purpose of this paper is to review available tissue clearing methods and justify the advantages of a particular protocol of tissue clearing named CUBIC over the other methods in imaging and 3D reconstruction. This method can be implemented at a relatively low price, does not require specialized equipment, and provides an opportunity to observe normal and tumor tissue samples in details.

MAJOR GROUPS OF TISSUE CLEARING APPROACHES

All tissue clearing methods aim to reduce light scattering by equalizing the refractive index between different substances in tissues (1). We can describe the tissue clearing methods according to the followingfeatures: use of chemical compounds for delipidation, depigmentation and decolorization, capability of preservation of fluorescent signals after clearing, clearing performance, and pricing of the equipment (Table 1).

According to chemical compounds we can introduce aqueous solution-based and non-aqueous solution-based clearing techniques (solvent-based clearing). The aqueous-based clearing techniques affect the tissues with dissolving of the lipids and refractive index matching (1). According to their mechanism of action, the aqueous-based clearing techniques are divided into three subtypes: simple immersion, hyperhydration, and hydrogel embedding (1) (Table 1). The non-aqueous solution-based clearing techniques are chronologically the first to be developed, they clear tissues through dehydration (1).

Although the idea of tissue transparency is more than 100 years old, various methods have only been developed in the last 15 years and have been well described in recent literature (3) (Fig. 1). For the very first time the tissue transparency approach was proposed by the German anatomist Werner Spalteholz. Back in 1914 he published an article with the clearing method technique discussed in steps needed to achieve the transparency of the tissue sections (4). According to Spalteholz's protocol, the main steps to follow are: formalin fixation and pigment-bleaching with hydrogen peroxide followed by a gradient dehydration, which is achieved via serial ethanol solutions and incubation in the clearing solution. With his article (4) Spalteholz published his clearing method and described the steps needed to achieve transparent sections based on reducing the refractive index, allowing light to penetrate deeper into the tissues. To date, with some minor modifications over the years, Spalteholz's protocol is still in use. During the last two decades, the development of methods for 3D reconstruction and the increasing computation power have predisposed the development of other tissue clearing methods (3). Spalteholz's innovative method has a deficiency associated with changes in tissue structure and therefore his technique has been

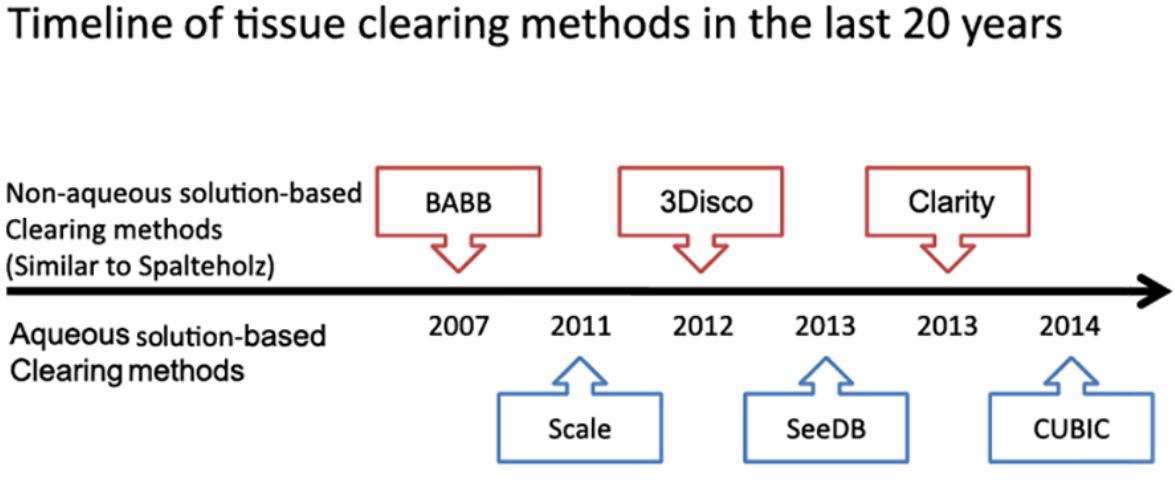


Fig. 1. Timeline of tissue clearing methods.

modified for the study of blood vessels in tissues (5). In order to achieve tissue clearing with minimal deteriorating effects on the tissue, other methods like CUBIC (6) have been developed.

A technique called benzyl alcohol and benzyl benzoate (BABB) was introduced based on the Spalteholz's protocol (7) (Fig. 1, Table 1). Benzyl alcohol and benzyl benzoate is useful for clearing the mouse brain and embryos, and whole small model organisms such as fruit flies (7). Although the method is inexpensive and the clearing performance is high, BABB uses toxic compounds and causes a high tissue shrinkage and a reduction of fluorescence signals, which are significant disadvantages (8).

The 3DISCO (3D imaging of solvent-cleared organs) protocol (9) is an alternative of BABB, because the substitution of benzyl alcohol/benzyl benzoate with DBE (dibenzyl ether) preserves fluorescence signals. Nevertheless, the disadvantages of this method are tissue shrinkage and the use of toxic compounds (8) (Fig. 1, Table 1).

The ScaleS (sorbitol-based optical clearing method) protocol, discovered by Hama et al. (10) in 2011, represents a tissue clearing method using an aqueous solution of uric acid. As the tissues are embedded in a hydrophilic environment, the fluorescence signals are preserved after the clearing steps (8). On the other hand, this aqueous reagent-based clearing procedure results in increased friability of the specimens due to the protein loss, which possibly alters the morphology (8) (Fig. 1, Table 1).

Another aqueous clearing method is SeedB (See Deep Brain) (11), used to clear mouse brains and embryos. This clearing procedure utilizes fructose-based solutions and preserves both the fluorescence signal and the morphology of the specimen (8). The disadvantages using the SeedB method include a variable timing of the procedure (hours to weeks) and a higher price compared to other methods (8) (Fig. 1, Table 1).

One of the tissue clearing methods with highest clearing performance is CLARITY (clear lipid-exchanged acrylamide-hybridized rigid imaging / immunostaining/ in situ hybridization-compatible tissue hydrogel) (12). The main disadvantage of CLARITY is the requirement of specific and expensive equipment like platinum-based electrodes for the electrophoresis (Fig. 1, Table 1).

THE CUBIC METHOD

CUBIC was introduced by Suzaki et al. (6) in 2014, its abbreviation stands for: *c*lear, *u*nobstructed *b*rain/*b*ody *i*maging *c*ocktails and computational analysis. CUBIC is an easy-to-use, low-cost histological method in which tissue samples are immersed in amino alcohols. The latter remove lipids from the tissues, which in turn reduces the refractory index and increases transparency. 3D reconstruction of translucent tissue is possible with the usage of both confocal microscopy and light sheet microscopy. 3D reconstruction methods help in better understanding the tissue structure.

Although multiple optical clearing methods are available nowadays, an advantage of the CUBIC technique is its compatibility with immunofluorescence staining. This allows immunohistochemical detection of multiple antigens in a complex tissue context. The CUBIC technique offers multiple variants of application. In particular, CUBIC can be applied using thin tissue specimens from various human organs and tissues (13) (2–5 μm), thick tissue specimens (over 100 μm, 200 μm, and 300 μm) (14) or even whole organ mounts as shown for mouse brains (6).

The basic steps of the CUBIC protocol are as follows: (*i*) formalin fixation; (*ii*) fixed tissues treatment with the Reagent 1 for several days allowing the tissue to become transparent (lipid and heme extraction), and (*iii*) immersion in Reagent 2 to increase transparency (by matching the refractory indices) of the tissues. In fact, the second step of CUBIC corresponds to the lipid bleaching step of the Spalteholz's protocol. After the satisfactory transparency of the tissue is achieved, Reagent 2 should be applied, followed by microscopic evaluation. In particular, light-sheet fluorescence microscopy allows a more accurate 3D reconstruction (6).

We show an example of a whole rat eye treated according to the CUBIC protocol. The achieved transparency can be evaluated macroscopically by comparison with a normal rat eye (Fig. 2).

As already mentioned, the concept of making tissues transparent for microscopy is a century old. Over the last 20 years, multiple improvements

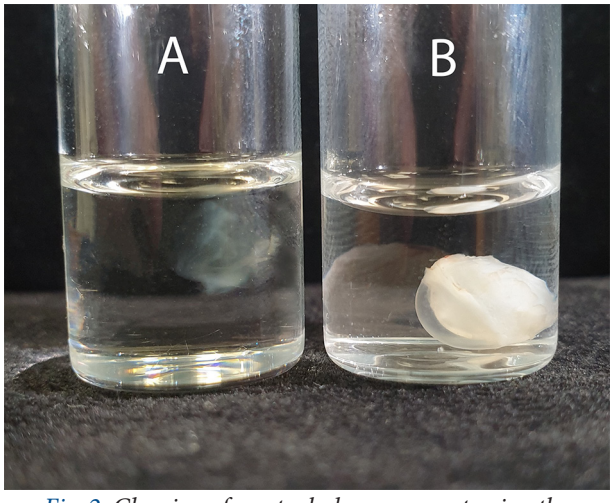


Fig. 2. Clearing of a rat whole-eye mount using the CUBIC protocol. Both specimens have a similar orientation (cornea facing to the left). The CUBIC-treated specimen (A), immersed in CUBIC 2 is almost completely transparent, while the control eye immersed in 30% sucrose-PBS for long-term storage remains with its typical opacity.

have been developed, including some variations of the Spalteholz's protocol, such as the methods BABB (7) and the 3DISCO (9), which use incubation of tissues in series of alcohols to achieve transparency. Despite the good results, these two approaches appeared to be quite aggressive towards fluorescent proteins, not allowing the subsequent immunofluorescence staining.

In contrast, CUBIC, along with the tissue transparency, offers the opportunity to achieve preservation of the immunofluorescence signal. Another advantage of CUBIC, unlike the CLARITY method, which does apply expensive equipment and reagents, is its cost-effectiveness.

CONCLUSION

CUBIC is an easy-to-use, low-cost histological method for tissue clearing. It minimizes the light scattering by achieving tissue transparency. In addition, this technique minimizes the absorption of light because the amino alcohols remove substances with high extinction (like heme) from the tissue. It has been shown that whole organ staining (e.g., mouse brain) and imaging is possible along with the 3D reconstruction of complex structures.

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