REVIEW ARTICLE

Three-Dimensional Approaches in Histopathological Tissue Clearing System

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조직투명화 기술을 통한 3차원적 접근

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ABSTRACT

Three-dimensional microscopic approaches in histopathology display multiplex properties that present puzzling questions for specimens as related to their comprehensive volumetric information. This information includes spatial distribution of molecules, three-dimensional co-localization, structural formation and whole data set that cannot be determined by two-dimensional section slides due to the inevitable loss of spatial information. Advancement of optical instruments such as two-photon microscopy and high performance objectives with motorized correction collars have narrowed the gap between optical theories and the actual reality of deep tissue imaging. However, the benefits gained by a prolonged working distance, two-photon laser and optimized beam alignment are inevitably diminished because of the light scattering phenomenon that is deeply related to the refractive index mismatch between each cellular component and the surrounding medium. From the first approaches with simple crude refractive index matching techniques to the recent cutting-edge integrated tissue clearing methods, an achievement of transparency without morphological denaturation and eradication of natural and fixation-induced nonspecific autofluorescence out of real signal are key factors to determine the perfection of tissue clearing and the immunofluorescent staining for high contrast images. When performing integrated laboratory workflow of tissue for processing frozen and formalin-fixed tissues, clear lipid-exchanged acrylamide-hybridized rigid imaging/immunostaining/in situ hybridization-compatible tissue hydrogel (CLARITY), an equipment-based tissue clearing method, is compatible with routine procedures in a histopathology laboratory.

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INTRODUCTION

Presently, the investigation of whole intact tissue

based on three-dimensional (3D) histology is available thanks to the modern advances in optical instrument, tissue clearing and novel molecular labeling techniques. Dense and tight texture formed by chemical tethering of hydrogel can enhance the preservation of molecular tissue components in addition to electrophoretic delipidation by which the opaque surface turns into

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transparent substance enabling clear observation of entire tissue sample even with naked eyes [1]. Three-dimensional reconstruction of intact tissue always gives comprehensive information for better understanding of structural and interactional whole data set which cannot be achieved by two-dimensional (2D) serial section slides due to the inevitable loss of information caused by unnatural artificial distortion: serial sectioning, of intact tissue [2]. Continuing efforts to explore the unseen world hidden by limitation of technology in tissue clearing, optics and computational 3D rendering, opened a new era of detailed explicit visualization of whole tissue imaging. Development of confocal microscopy and relevant optical advancement allowed researchers to access high resolution images acquired along the z-axis direction in a precise step dividing by several hundred nanometers. However, the benefits gained from the objectives with long travel range and a high power laser with optimized beam condition are naturally diminished because of the light scattering phenomenon and that is incremented by refractive index (RI) mismatch of each cellular component [3]. In history, there have been many attempts to overcome this physical hindrance by matching RIs, replacing body fluid with plastic monomer, hyperhydration with aqueous-based clearing solution and tissue-gel hybridization followed by electrophoretic lipid removal [4]. Autofluorescence is one of the matter should be concerned in order to make sure of clarified visualization with much reduced interference from unwanted fluorescent signals following the tissue clearing [5]. Bleaching of the tissue is often requested before tissue clearing to get rid of endogenous fluorescent biomolecules or autofluorescence acquired during fixation, staining process and sample preservation with diverse chemical reagents [6-8]. Immunolabeling of target molecules is a mandatory histotechnical step for microscopic visualization unless otherwise coupled by genetically modified fluorescence sequences. For large-scale biological tissues, specialized techniques adopting alternative immunofluorescent labeling process progressed in

epitope binding affinity, prolonged labeling time under stable condition, increased permeability for deep traveling of antibodies and multi-round and multiplex fluorescent labeling for follow-up studies, should be considered. Moreover, the several challenges and technical limitations of current tissue clearing methods have should be also overcome by continuous endeavoring of searching for the proper answers: how to deal with tremendous image data, non-economical time consuming procedures, deformation of original intact tissue structure and other procedurally associated risks when applied to clinical samples. In this review, a vast scope of tissue clearing techniques from the historical background, comparison of 2D and 3D image analysis, autofluorescence eradication, effective immunolabeling and RI matching to presently introduced cutting-edge tissue clearing methods is expatiated for better understanding of CLARITY to medical technologists engaged in the histopathology laboratory.

MAIN ISSUE

1. History of tissue clearing

The purpose of tissue clearing is to make the objects transparent without morphological denaturation which may cause misunderstanding of intact natural environment of tissue sample, in order to achieve comprehensive three dimensional constitution of tissue sample [9]. The 3D visualization of molecular localization and interactions without distortion by serial section of sample tissue allows medical technologists to explore the realm of hidden nature that contemporarily used conventional 2D imaging techniques in histopathology may barely impart. The first approach of tissue clearing was attempted by Spalteholz, a German anatomist [10], after realizing that contemporary histological methods were no longer effective for his new discoveries in macroscopic structures [11, 12]. The clearing solution invented by Spalteholz was a crude mixture of organic solvents: methyl salicylate and benzyl benzoate, in the wintergreen oil so as to match RI to 1.55 [13]. The

protocol could be briefly condensed with several steps: formalin fixation, pigment bleaching with hydrogen peroxide, gradient dehydration by serially increased ethanol concentration and incubation in the clearing solution [14]. This method, however, rarely introduced due to its incremented damages on the superficial layers of the tissue up to few centimeters as well as tissue shrinkage besides the limitations which solvent-based clearing protocols mainly have: a) tissue transparency, b) immunolabeling and c) endogenous fluorescence stability [15]. Canada balsam is, a hydrophobic oleoresin found in the bark of the fir *Abies balsamea*, widely used as a transparent mounting media in histopathology laboratory due to its very close (RI: 1.53) to typical crown glass (RI: 1.52), after the tissue dehydration and clearing with xylene. However, the strong autofluorescence that Canada balsam intrinsically bears disturbs clear observation of immunofluorescence labelled tissue sample, unlikely routine histochemistry staining. Other disadvantages reported were color changes to yellow after long deposition, becoming acidic over time, poorly preserved cationic dyes and bleaching of Perls' Prussian blue products [16]. More recently, plastination has been introduced for long term preservation of tissues, organs, systems and whole body by replacement of water and lipid with plastic monomers which impart transparency to the objects [17]. Plastination was rather suitable for establishing educational materials than used for biomedical research in histopathology due to its penetrating limitation less than 3 mm of thickness after plastic polymerization [18-20]. As the optical sectioning instruments: confocal laser scanning microscope (CLSM), multi-photon microscope, selective plane illumination microscopy (SPIM) and light sheet fluorescence microscopy (LSFM), had been consecutively developed for the last two decades, travelling into the deep tissue without physical sectioning of intact tissue sample was also available by virtual 3D image reconstruction based on the fluorescence labelling techniques and image rendering software:

Imaris (Bitplane, Belfast, UK: <https://imaris.oxinst.com/learning/view/article/researchers-combine-tissue-clearing-with-3d-rendering-to-reconstruct-bones,-organs,-and-brain-of-whole-mouse>), Volocity® (Quorum Technologies Ltd, Laughton, UK: <http://quorumtechnologies.com/volocity/volocity/restoration>), Amira (Thermo Fisher Scientific Co., MA, USA: <https://www.fei.com/software/amira>) and Huygens (Scientific Volume Imaging B. V., Hilversum, Netherlands: <https://svi.nl/Huygens-References>) [21, 22]. However, the scattering of light from the heterogeneous cellular contents such as nucleus, mitochondria, ribosome, cytoskeleton and membrane during excitation prohibits qualified imaging acquisition in thick tissue deeper than 500 µm unless otherwise pretreated with tissue clearing solution for RI matching [23, 24].

2. Limited 2D image and advantage of 3D reconstruction in histopathology

Histopathology is the microscopic investigation of serially sectioned thin tissue subjected to the appropriate staining process: histochemistry, immunohistochemistry and immunofluorescence, etc., for clear visualization of physical properties, highlighting important features for diagnosis and enhancing tissue contrast by coloring of target molecules depending on the experimental purpose [25, 26]. Microscopic slides prepared by medical technologists in clinical pathology laboratory contain a vast amount of information under the magnification. However, they have to select few confined typical fields experimentally for the clinical report from the prepared series of tissue slides out of whole tissue block from patients. Such stochastic choice of evidence out of serially sectioned lesion not from intact tissue itself sometimes seems scarce in scientific rigor in comparison with other branch of life science and biology. Multimodal image registration is a multiplexed imaging technique which combines simultaneously produced signals from different modalities such as multi-channel 2D images, differential interference contrast (DIC), phase contrast or electron

microscopy (EM). Image registration is the process of transforming different sets of data into one coordinate system and the data may be multiple photographs, data from different sensors, times, depths, or viewpoints. Multimodal image registration followed by 3D reconstruction using multi-channel 2D images of histopathology enables demonstrating the volumetric anatomical dataset which embodies spatial distribution of target molecules, topographical localization of lesion and functional structures of patient originated sample tissue [27]. However, regulation traditionally lags behind innovation when it comes to the current circumstances in the US. The Food and Drug Administration (FDA) currently approved the digital slide scanner and its automation system in histotechnology for the use of diagnostic purpose in the US although it had been already adopted among European societies for diagnostic, educational and research purposes [28]. Whole slide image (WSI) can be acquired by optical scanning of glass slide, tile by tile or in a line-scanning fashion. Adoption of WSI for the day-to-day practice of surgical pathology, teleconsultation and routine diagnosis in histopathology appeared to be successful in few cases and the needs for the application would be gradually increased as time goes by [29]. On the other hand, there have been consecutive efforts to cross the borderline of adopting 3D histopathology as a routine method for clinical research. Three-dimensional reconstruction of tissue at microscopic resolution in histopathology imparts an abundance of functional information through histochemical staining, immunohistochemical conjugating and immunofluorescent multi-channel labeling unlike other 3D computational modeling based on magnetic resonance imaging (MRI) or micro CT image. Recently, there was a pioneering attempt to build a multi-parametric 3D representation by alignment of volumetric data from quintuple staining 3D images with high resolution MRI in order to provide further anatomical information. The main purpose of this experimental attempt was to provide multi-scale physics based

models based on the anatomical and functional data acquired from rich data set [30]. Indeed, the digital pathology system including 3D reconstruction of microscopic multi-channel images provides alternative way to gain a comprehensive understanding of whole tissue sample and insight on the specific lesion of whole tissue image.

3. Currently used tissue clearing methods, and elimination of autofluorescence background from fluorescence labeled tissues

Nowadays, various tissue clearing techniques and RI matching materials are selectively used depending on the composition, condition, size and type of sample tissue in order to achieve the successful tissue clearing which preserves intact state of sample tissue at the moment of extraction and guarantee the integrated image acquisition. Basically, most of the tissue clearing methods launched recently are composed of four major steps: tissue pretreatment, permeabilization or delipidation, immunolabeling and RI matching as a final clearing procedure unlike some of the early developed methods which were comprised of fewer steps: exposure to the clearing mixture and RI matching [31]. Bleaching of the tissue is often requested before tissue clearing to get rid of autofluorescence acquired during fixation with formaldehydes and prevent absorption of light from endogenous fluorescent biomolecules: NADPH, collagen, flavins, porphyrins, tyrosine, lipofuscins and chlorophyll in plants, usually on the vertebrate skin, kidney and liver [5]. Mostly, the emission spectrum of autofluorescence is wider than the labeled fluorescent dye or exogenously inserted fluorescent proteins, and that causes predicament in separating the target fluorescence from unwantedly dominant autofluorescence in the sample. The endogenous autofluorescence, however, mostly disappeared on the paraffin section during fixation and dehydration with many different organic solvents. On the other hand, optimal cutting temperature (OCT) compound which consists of 10.24% polyvinyl alcohol, 4.26% polyethylene

glycol and 85.5% of non-reactive ingredients, for the frozen section doesn't seem to reduce the endogenous autofluorescence compared to the paraffin section. Lipofuscin, which is mainly observed in neuron and retina cells under wide range of excitation spectra around 420~633 nm even after complete exposure to the organic solvent during fixation and dehydration of paraffin section, is the fine yellow-brown pigment granules made up of oxidized proteins and lipid containing residues with sugars and metals: mercury, aluminium, iron, copper, zinc, etc., created by breakdown process of unnecessary components of cells via lysosomal digestion [32]. The accumulation of lipofuscin in tissue gets normally increased by aging, and in few cases, the impaired balance between formation and degradation mechanism of lipofuscin results in augmented autofluorescent clusters in cells [33]. Extrinsic autofluorescence, observed in yellow color through the wide field microscope with long pass filter, is experimentally triggered by aldehyde fixatives and its cross-linking reaction between amino groups of lysine residues and interrupts microscopic observation of target fluorescence labeled with Alexa 488 shown in glowing green. In order to get rid of fixative-induced autofluorescence, a number of approaches: a bleaching technique using light emitting diodes (LEDs), chemical agent treatment which eliminates Schiff's bases and spectral unmixing method, have been come up with. The photo-irradiation using an array of LEDs: 390 (UV), 430, 460, 630, 660 and 850 nm (IR) to the deparaffinized and free floating vertebrate cerebral cortex tissues for 2 hours effectively quenched and reduced fixative-induced artifactual autofluorescences by 88% compared to the paraffin-embedded which showed slower bleaching process by 31%, 67% and 84% after 2, 4 and 24 hours of exposure to LEDs [34]. Another intriguing method to avoid the interference from unwanted autofluorescence is to use an alternative protocol which adopts use of minimal concentration of aldehyde. Tokumasu and Dvorak introduced the autofluorescence compromising fixation protocol for immunocyto-

chemistry of erythrocyte fixation, using dimethyl suberimidate (DMS); diimidoester cross-linking agent, with 2% paraformaldehyde (PFA) so that aldehyde-amine reaction; the main cause of fixation-induced autofluorescence, could be reduced [35]. The spectral unmixing is the computational spectrum decomposition technique for separating overlapping emission spectra from multiple fluorophores including autofluorescence [36]. The diffraction grating and multiple-anode photomultiplier tube (PMT) are needed for dispersion of wide range of emission colors and multichannel detection within narrow spectral bands around 10~20 nm. The emission spectra based on the acquired image are subjected to software analysis "unmixing" which enables separating the target fluorescent signal out of autofluorescence; however, the intensity peak of target signal should exceed the maximum intensity of autofluorescent spectrum for its clear separation. Other methods commonly used for quenching autofluorescence induced by unreacted aldehyde groups are glycine, sodium borohydride, ammonium chloride, sudan black B, cupric sulfate, trypan blue and ethanol in addition to recently introduced instrument based or optical physics oriented autofluorescence quenching methods: fluorescence lifetime imaging (FLIM) via time-gated detection or LED photo-irradiation bleaching (Table 1). The time-gated detection for FLIM utilizes the pulsed laser: femtosecond laser which emits optical pulses shorter than 1 picosecond, and a detector with optical chopper in order to measure the lifetime of a fluorophore that spans hundreds picoseconds to nanoseconds, for instance, cyanine3 (Cy3) shows 300 picoseconds for decaying; on the other hand, Alexa Fluor 488 by 4.1 nanoseconds (Table 2). The discrete lifetime of autofluorescence and labeled fluorophores is the key point for extracting the real signal of target molecules out of dominant autofluorescence. Above all, eradicating unwanted autofluorescence of the tissue is not always naturally achieved by a series of tissue clearing methods depicted in this review, but the apparent bleaching procedures should be accomplished

for much clearer observation of the target molecules shrouded by autofluorescence. The clearing methodologies presently used are roughly divided into three groups: aqueous-based RI matching by simple immersion, aqueous-based hyperhydration and tissue-gel hybridization. For simple immersion, equivalently matching the optimal RI of the sample tissue by soaking in the high RI medium enables transparency of the tissue although the technique is naturally slow and passive under simple aqueous solution. Most simple immersion methods economical and easy to apply to the laboratory experiments since those are quite compatible with a variety of commercially available fluorescent dyes and importantly do not remove lipid, and that trait allows the membrane targeting investigation. Usually, high concentration of sucrose or other aqueous combination containing several molecules with high concentration are used as a RI medium for simple immersion clearing. Most of the aqueous-based clearing media have typical RI values around 1.38~1.48 and those are not always synchronized with oil immersion (RI: 1.52) objective lenses on the light microscopes and glass coverslips (RI: ~1.52). The RI mismatches between the sample tissue and objective lenses cause spherical aberration and impaired z-axis resolution with lowered image quality [37]. *Scale* is the most well-known aqueous-based hyperhydration clearing method which uses nonionic detergents: Triton X-100, instead of hydrophobic solvent for removing lipid, and also contains a diverse combination of urea and glycerol as clearing solution. The urea containing aqueous solution commonly manifests similar RI to water (RI: 1.33) and this trait facilitates microscopic investigation of thicker tissue using water-immersion objectives which have longer working distance compared to the oil immersion objectives [38]. There are several types of *Scale* formula lately improved for each purpose of usages: *ScaleA2* (4 M urea, 10% glycerol and 0.1% Triton X-100), *ScaleU2* (4 M urea, 30% glycerol and 0.1% Triton X-100) and *ScaleS* (20% sorbitol, 5% glycerol and 0.2% Triton X-100). Both urea and sorbitol have effective tissue clearing properties;

however, urea incurs hyperhydration which leads to the tissue swelling as clearing proceeded. On the other hand, replacement of urea by sorbitol causes tissue shrinkage in *ScaleS* method [39]. Although the outline of tissue is swollen or shrunk depending on the *Scale* methods applied, the overall tissue shape and internal structures do not seem too much distorted from the original shape of the sample tissue. However, those methods intrinsically have a critical drawback that longer incubation time is needed for complete tissue clearing up to several weeks compared to the tissue-gel hybridization also known as "CLARITY".

4. CLARITY

Over the past decades, multifarious experimental approaches for novel tissue clearing methods had been attempted. The constant needs for the satisfactory tissue clearing technique, which rapidly transforms sample tissue into an optically transparent and macromolecule-permeable structure for significantly improved immunolabeling and much reduced time consuming while simultaneously preserving its native molecule information without physical denaturation, led to the advent of CLARITY. Delipidation of mammary glands by solvent-based clearing solution causes the demolition of lipid barriers, so that antibody for immunolabeling can travel into the every corner of cells. The extreme dehydration triggered by the clearing solvents is, however, connected to the removal of water molecules in cells and that phenomenon is usually followed by the unstable state for fluorophores apart from their quenching during clearing process [40]. Moreover, high concentration of detergent or prolonged exposure time to the toxic hydrophobic solvents are intrinsically vulnerable to the loss of protein contents of the cells, and the aqueous-based clearing methods are generally optimized for the small size tissue [41]. On the other hand, CLARITY stably removes lipid under less harmful hydrophilic environment; protects intact ultrastructure of the sample tissue by tissue-gel hybridization, in company with well-preserved

endogenously expressed fluorescent proteins; and increases accessibility of macromolecules such as antibodies and nucleic probes to target epitopes and complementary sequences [42]. Lipid bilayers of cell membranes are subjected to removal completely for successful tissue clearing; however, the scaffold of the cell membranes should be replaced by another sustainable framework to circumvent sudden collapse or irreversible distortion of sample tissue. For tissue-gel hybridization, sample tissue should be incubated at 4°C in the mixture solution of hydrogel monomers (mostly, acrylamide and bisacrylamide), formaldehyde and thermal initiator that passively infuses into every corner of the tissue. The air bubbles and residual oxygen, which impedes hydrogel formation in the tissue, should be removed and that can be achieved by using vacuum pump or desiccation chamber with nitrogen gas. Inappropriate removal of air bubbles may result in opaque body with lots of cavities after lipid extraction. According to the latest report, thick layer of mineral oil added on the top of the mixture solution of hydrogel monomers reduces likelihood of exposure to the air and extrinsic originated bubble formation before polymerization [43]. The formaldehyde crosslinked with amine-containing tissue components also covalently links the hydrogel monomers to native biomolecules such as proteins, nucleic acids and other cellular small molecules. After the hydrogel polymerization which is thermally initiated at 37°C for 3 hours in the case of mouse whole brain, the cellular lipid (a major hindrance of antibodies penetration for immunolabeling and photon traveling throughout the entire tissue) is dissolved by ionic detergent-based clearing solution (borate-buffered 4% SDS) without immediate consequential collapse owing to the preliminarily hybridized gel scaffold. Dissolved lipids forms plenty of SDS micelles which are highly negatively charged at pH 7.5~8.5 due to the ionic detergent in sample tissue, and are actively extracted by electrophoretic tissue clearing (ETC) system minimum for 6 hours up to several days depending on sample size, lipid content ratio and

proportion, and specification of ETC equipment (Figure 1). The electric field strength should be carefully controlled by reckoning the sample size and lipid content thereof. The continuous electric power input with high voltage to ETC chamber for electric field formation is mainly connected to overheating the ETC solution and tissue deformation which results in the significant denaturation of epitopes and endogenous fluorescence quenching [44]. Even though warmer operating temperature and higher electric power voltage are relevant to the fast clearing of the tissue, it is experimentally recommended that circulating clearing solution in the ETC chamber are needed to stay at 25 V and 37°C for effective tissue clearing without loss of fluorescence and target epitopes [45]. In addition, the speed of clearing and efficiency of immunolabeling is also critically affected by the consistent nanoporous structure and pore size formed during hydrogel polymerization. The hydrogel formula out of formaldehyde and acrylamide is the decisive factor for hydrogel mesh density. A dense hydrogel mesh made of higher concentration of formaldehyde and acrylamide gives hardened enough solidity to the tissue but loses pros of CLARITY such as speedy tissue clearing and smooth antibody penetration. On the other hand, soft hydrogel mesh with lower concentration of hydrogel mixture usually ended up with tissue crumbles after delipidation. Therefore, well balanced rigidity and porosity given to hydrogel mesh by optimized combination ratio of the hydrogel mixture solution are highly requested for the successful tissue clearing in many different tissues and immunolabeling thereof [46]. Hydrogel frame endows secured environment for biomolecules and fine structural features: membrane-localized proteins, synapses, filaments and nucleic acids, whereas it facilitates smooth removal of membranous lipids, the main cause of light scattering and diffraction during microscopic investigation and major obstacle of macromolecules penetration for immunolabeling in deep tissue. In the aspect of preserving native proteins, CLARITY showed improved

capability in preserving innate molecules by 8% protein loss compared to conventionally paraformaldehyde-fixed tissue blocks cleared by 4% SDS for 1 week showing ~65% protein loss; Scale, an urea based clearing method with 41% protein loss; and a mild detergent-based permeabilization solution with 0.1% Triton X-100 showing 24% protein loss [47]. Conversely, systematically varied acrylamide/bisacrylamide/formaldehyde combination for hydrogel embedding and quantification of protein contents by Bradford assay and SDS-PAGE analysis exhibited a poor correlation between the hydrogel combination and the amount of protein loss [48]. Considering both aspects: quite variable protein loss depending on the methods for clearing and poor correlation in protein quantification as per different combination and formula of hydrogel mixture, it's thought that the consequential protein loss is somehow related to the prolonged exposure time to the clearing solution and rate of lipid dissolution out of the sample tissue apart from the stable molecular tethering to the hydrogel network structure. More recently, there has been an attempt to introduce CLARITY to histopathology for a clinical diagnosis by providing the evidence that CLARITY could be integrated into a standard clinical workflow due to its compatibility with frozen section and formalin-fixed human specimens. Indeed, volumetric histology and 3D digital pathology are the terms coined nowadays for emphasizing the usefulness of tissue clearing in clinical pathology now that three-dimensional techniques: tissue-gel hybridization, delipidation, fluorescence multilabeling, RI matching, 3D imaging, deconvolution and 3D image rendering for data analysis, allows transparent volumetric substance with a computational quantitative analysis out of the convoluted opaque morphology of sample tissue in which those are not normally acquired by traditional section-based histopathology such as low-grade abnormalities in glandular cell growth, 3D visualization of angiogenesis within tumors, ductal integrity and morphology and 3D tumor invasion into surrounding tissues [49]. However, endeavoring for

additional improvement of CLARITY such as shortening exposure time to detergent and clearing chemicals, much more optimized tissue-gel hybridization with firm and strong structure to avoid tissue crumbling after the hybridization and antibody deep penetration into every corner of tissue for the perfect labeling with fluorescent dyes, is also requested. Compared to other methods, CLARITY is the only equipment-based tissue clearing method meaning the higher compatibility to the present workflow in the clinical laboratory where end-users mostly prefer the automation for sample preparation, and increased productivity and efficiency in processing multiple tissue samples obtained simultaneously by integrating to the laboratory routine procedure.

5. Tissue labeling

Immunolabeling is a fundamental visualization technique by which molecular localization and interaction can be precisely understood with comprehensive perception on microworld. The noble immunolabeling technique following CLARITY for large-scale biological tissues allows much improved clinical investigation with the abundant of structural and molecular information deeply related to the diseases without artificial deforming and disassembling the sample tissue subjected to serial section during the sample preparation in routine histopathology procedure. In general, immunofluorescent labeling with antigen specific molecular probes is conducted under natural condition of diffusion which takes longer time and slower speed when it comes to the simultaneous labeling of multiple targets in huge scale tissue sample. Multi-round and multiplex fluorescent labeling in the large volume of cleared tissue are technically restricted although there have been enormous efforts to overcome the limitation by continuous attempting to search for the breakthroughs such as improving permeability throughout entire large volume of tissue, exerting artificial flow and external force to facilitate penetration of probes into every corner of the tissue, and increasing

probe sensitivity and specificity by modulating probe-targeting binding kinetics [50]. However, the increased structural and chemical stability under hydrogel context in CLARITY enables multi-round molecular phenotyping up to three times using the ionic detergent (4% SDS/neutral-pH buffer) which is adopted for stripping out antibodies and disrupting binding affinities thereof, unlike previously used antibody elution method which is usually implemented under acidic buffer with potassium permanganate (KMnO_4) oxidation or exposure to extreme heat where major structural damage, degradation of antigenicity and endogenous fluorescence quenching consequentially occurred [51–53]. The efficiency of tissue clearing is affected by the fixation procedure and moderately reduced hydrogel density which forms networks along the formaldehyde crosslinking is related to the antibody penetration deeper into every corner of the thick tissue spanning around 30 to 100 μm of thickness. It is presumed that hydrogel infusion without bis-acrylamide results in less protein-acrylamide crosslinking and that imparts multi-porosity with increased permeability to the sample tissue. The loosened structure of cleared tissue is vulnerable and fragile in terms of keeping its intact shape and preserving native proteins; however, it facilitates rapid extraction of lipid and invasion of macromolecules deep into the sample tissue. More recently launched labeling method, PRESTO (pressure related efficient and stable transfer of macromolecules into organs), adopted the centrifugal force or pressured convective flow in order to maximize penetration efficiency of macromolecules deep into the dense tissue over 1 mm in size [54]. The time consumed for immunostaining of cleared mouse kidney optically sectioned and stacked by 200 μm was notably reduced by 3 hours in both c-PRESTO (centrifugal) and s-PRESTO (syringe) with extremely improved antibody penetration of collagen type IV and nucleic acid labeling with SYTO16 dye compared to the passive staining method by free diffusion [55]. Interestingly, those active immunolabeling methods using impact

convective flow or mechanical force manifest several negative phenomena such as signaling saturation incurred by over-staining despite of short labeling time, and intensified background signals due to nonspecific deposits of macromolecules accelerated by artificially introduced strong stream and insufficient rinsing process for PRESTO.

6. Refractive index

Perfectly cleared whole-tissue and whole-body imaging out of opaque tissue sample is mainly dependent on the successful delipidation process which at the same time also preserves heterogeneously inserted fluorescent proteins satisfactorily, and that can be achieved by adopting the sophisticated tissue clearing methods depicted above. A modified immunolabeling technique which demonstrates improved travelling abilities of macromolecules deep into the every corner of completely clarified sample is also indispensable aspect for significantly enhanced contrast and stretched z-axial realm during image acquisition. Although ultimately transparent object thoroughly labeled by antigen specific macromolecules is the undeniable core procedure of tissue clearing, the importance of RI matching of cleared tissue is also doubtless due to the characteristics of light, mainly scattering and refraction. The common obstacle of optical imaging of cleared tissue is light scattering showed along the entire 3D tissue. Each component of cellular and extracellular structures combined with experimental materials applied for sample preparation demonstrate various RIs [56]. The RIs of various proteins in cells can be presumed by calculating the refraction value of discrete compositions and residues of amino acids [57]. Heterogeneously distributed cellular components: various amino acids, proteins, nucleic acid and related substances, are spread throughout the post-tissue clearing sample and attributed to photon scattering during excitation. Recently, there have been continuous efforts to come up with RI matching medium universally optimized by mixture of multiple chemical

Table 1. Feasible approaches for elimination of artifactual autofluorescence background

Method	Autofluorescence elimination efficiency	Drawbacks	References
Time-gated detection method combined with FLIM and ADOA fluorophore	Excellent	Access to the equipment and operation is complicated compared to other approaches	[60, 61]
LED or laser-induced photobleaching	Excellent	Delayed exposure time for thicker tissue sample	[62, 63]
Sudan black B (SBB)	Good at 0.1% of SBB in frozen section Moderate at 0.1% of SBB in paraffin section	SBB also dissolves many types of lipids on the tissue	[64, 65]
Cupric sulfate (CuSO ₄)	Moderate	Likelihood of reduction of the intensity of specific immunofluorescent labeling	[6, 66]
Sodium borohydride	Moderate	Chemically reduce C=C and C=N bonds and diminish the autofluorescence; however, unstable at neutral pH and poor reproducibility	[7, 67]
Trypan blue (TB)	Moderate in pretreatment before paraffin embedding Poor in posttreatment after immunolabeling	Posttreatment after embedding in paraffin wax emits red fluorescence in retinal pigment epithelial	[68, 69]
Ethanol (70%)	Moderate in frozen section Poor in paraffin section	Fixed lipid and lipofuscin by formaldehyde are barely dissolved by 70% ethanol	[70, 71]

Abbreviations: FLIM, fluorescence lifetime imaging; ADOA, azadioxatriangulenium; LED, light emitting diode.

Table 2. Fluorescence lifetimes of universally used fluorophores with excitation and emission peak [72]

Fluorophores	Lifetime (ns)	Quantum yield (φ)	Excitation peak (nm)	Emission peak (nm)	Source (ThermoFisher)
Alexa Fluor 488	4.10	0.92	490	525	A10235
Alexa Fluor 555	0.30	0.10	555	580	A20174
Alexa Fluor 594	3.90	0.66	590	617	A10239
Alexa Fluor 647	1.00	0.33	650	665	A20173
Cy3	0.30	0.15	554	568	M30010
Cy5	1.00	0.27	649	666	A10525
Rhodamine B	1.70	0.97	562	583	R648MP

ingredients. Originally, FocusClear (RI; 1.45, CelExplorer Labs, Taiwan), a water-soluble clearing agent improving the transparency of biological tissues less than 500 μm in depth from the tissue surface, was adopted for matching the average RI of biological samples (RIs; 1.35~1.55). Most of the commercially available RI matching medium arbitrarily prefers to tune its RI in that range since the RI of water, a major component of mammalian tissue, is (RI; 1.33) and other cellular organelles such as cytosol, nucleus, mitochondria and lysosomes are less than (RI; 1.60) (Table 3) [58]. Alternatively, FocusClear can be replaced by 85% glycerol (RI; 1.45) but tissue transparency achieved by FocusClear is much clearer than glycerin-water solution for short-term storage after RI matching. Interestingly, cleared tissue is slowly turning to opaque and unclear

with white pigment deposition in FocusClear after long-term storage. RI matching medium also can be formulated as a mounting cocktail in order to obtain best quality of transparency. The CUBIC-mount, a mounting cocktail in ACT-PRESTO, includes 25% of aminoalcohols (2-hydroxypropyl ethylenediamine) for bleaching; 50% of sucrose and 25 % of urea for adjustment of RI [55]. The RIMS (refractive index matching solution; RI; 1.47), a mounting solution of Histodenz (40 g) in 0.02 M PBS (pH 7.5) with 0.01% of sodium azide, is suitable for long-term storage of cleared tissue labeled with macromolecules. For cost effects, sorbitol RIMS (sRIMS), a combination of 70% of sorbitol in 0.02 M PBS (pH 7.5) with 0.01% of sodium azide, can be manually made by expending 0.2 USD per milliliter [38]. Simply, PBST (0.1% Triton X-100 in PBS)

Figure 1. CLARITY system and tissue clearing process with example images of mouse ovary. Mouse ovaries from young (7 weeks) were obtained and fixed with 4% paraformaldehyde (PFA). Ovaries were processed for histological observation and CLARITY. Fixed ovaries were immersed with hydrogel solution of 4% PFA, 4% acrylamide, 0.05% bis-acrylamide, 0.25% VA-044 (2,2'-azobis[2-(2-imidazolin-2-yl)propane]dihydrochloride), 0.05% Saponin and PBS at 4°C for 24 hours. Then, tubes containing ovaries were de-gassed to remove oxygen and polymerize hydrogel monomers at 37°C for 3 hours using ETC system; X-CLARITY™ (Logos Biosystem, Korea). Polymerized ovaries were transferred into 1 L of clearing solution containing 200 mM boric acid and 4% SDS dissolved in distilled water with addition of NaOH to reach pH 8.59. Delipidation was accomplished by electrophoresis under 1.0~1.5 A, at 37°C for 16 hours. The clearing solution was automatically circulated through the buffer drain controlled by peristaltic pump in the ETC system and ovaries gradually became transparent after 8 hours depending on sample size. (A) A polymerization system accelerating hydrogel infusion into the corner of the sample tissue under vacuum condition before electrophoretic delipidation. A hydrogel polymerized tissue is ready for delipidation in the electrophoresis chamber where lipid micelles are actively removed along the electric field under the electric current around 1.0~1.5 A at 37~45°C consecutively, for 16 hours in the case of mouse ovary. (B) Formaldehyde in a fixed tissue is cross-linked with hydrogel. Electrophoresis of the polymerized tissue allows charge dependent lipid removal via SDS micelle. Proteins and DNAs remain in the cleared tissue as conserved 3D networks. (C) Before and after tissue clearing of mouse ovary.

can be used as per the entire clearing procedure: washing, antibody dilution and immersion for matching RI; however, several drawbacks still remain such as poor transparency and over swollen of the tissue [59]. Even though the RI inhomogeneity of biological tissue is removed by the tissue clearing followed by treatment of RI matching medium, the RI mismatch between objective lens and cleared tissue still remains and that spawns an imaging issues; distortion of point-spread function (PSF). To this end, all the optical elements

Table 3. Refractive indices of biological components and optical materials for tissue clearing [73-75]

Biological components	Refractive index	Optical materials	Refractive index
Cytosol	1.36~1.39	Coverslip glass	1.52
Nucleus	1.36~1.37	Glycerol (85%)	1.45
Nucleolus	1.38~1.39	Glycerol (98%)	1.47
Mitochondria	1.40~1.42	Oil immersion	1.52
Lysosome	1.60	Water (DW)	1.33

between specimen in the RI matching medium and front lens of the objective should be ideally optimized by adopting a motorized correction collar which is adjustable depending on the RI of specimen for deep tissue imaging. Lastly, longer working distance (WD) around 6 mm with higher numerical aperture (NA) around 1.00 is a critical standard for ideal objective in tissue clearing.

CONCLUSION

Digital pathology system including 3D reconstruction of microscopic multi-channel images obtained by high-throughput and automated fluorescent microscope made it possible that consecutive capturing, precise stacking and accurate reconstructing entire tissue on the slide glass practically. A volumetric anatomical

dataset containing spatial information of target molecules and topographical localization of lesion provides comprehensive understanding of whole sample tissue which cannot be satisfactorily achieved by artificially sectioned multi-channel 2D images from intact whole tissue (Figure 2). Novel optical clearing methods used during the twentieth century were mostly based on organic solvents to reduce not only light scattering but also maximize imaging depth in intact tissue. Biological specimens made up of multiple cellular components with different RIs hinder clear observation under wide-field microscope. RI mismatches among multiple cellular components, liquid immersion and mounting solution severely cause scattering of light, chromatic aberrations and PSF distortion [76]. Furthermore, reduced signal intensity, poor image resolution, diminished contrasts and limited penetration

Figure 2. Example of three dimensional approach in histopathology. Serially sectioned sample tissue can be individually imaged under wide-field microscope with motorized stage for z-stacking which is followed by regeneration of XYZ-section image for presumption of three dimensional information by interrogating XZ and YZ plane view. On the other hand, a sample tissue can be solely imaged after tissue clearing without sectioning step artificially given under current histotechnology. Cleared ovaries were transferred into 15 mL conical tubes containing PBS with 0.1% Triton-X before serial rinsing with PBS for 1 hour to remove residual SDS. After which, samples were incubated with primary antibodies (anti-Mullerian hormone; red and platelet endothelial cell adhesion molecule-1; green) for 24 hours, and then washed with buffer for 1 hour, followed by fluorescent secondary antibodies for 6 hours and nuclear staining with 4',6-diamidino-2-phenylindole (DAPI; blue). Intact ovaries were mounted on the glass bottom dishes (WillCo Wells, Amsterdam, Netherlands) using FocusClear solution (CelExplorer Labs, Hsinchu, Taiwan) for RI matching. Transparent tissue with successful immunofluorescent staining was subjected to confocal imaging for 3D reconstruction and image analysis. Images were obtained using Leica TCS SP8 confocal microscope (Mannheim, Germany). Two- and three-dimensional images, as well as data analysis were obtained using Spots and Isosurfaces function based on the Imaris algorithm (Bitplane, UK).

depth of excitation light due to spherical aberrations could be incurred by RI mismatches of cellular components [77]. However, most of the organic solvent oriented tissue clearing methods invented still in its infancy were barely utilized owing to irreversible severe damages on the superficial layers of tissue that incurred critical deformation and shrinkage. To this end, various attempts with adaptive feasible techniques were made and eventually, novel clearing methods have been evolved by presenting solution for lipid bilayers impermeability, limiting penetration of macromolecules for whole mount staining methods and reduced stability of the tissue after clearing. The permeable nanoporous hydrogel mesh created by CLARITY following delipidation allows macromolecules to freely penetrate and rapid diffusion of antibodies and other probes for nuclear acid into deep inside of the tissue. Moreover, CLARITY allows multiple rounds of molecular phenotyping for target protein investigation, in situ hybridization after ensuring stable retention of RNAs in cleared human tissue for detection of microRNAs and mRNAs, and DNA-based fluorescent signal amplification of target mRNAs with hairpin chain reaction (HCR) amplification system [78]. Therefore, systemic investigation of many molecular phenotypes of protein complexes, gene expression profiles, and neuronal circuits is available in human tissue [79]. Another big consideration which medical technologists should give in for successful tissue clearing is “autofluorescence” and appropriate “antidote” in terms of reducing unexpected extreme background glow originated from endogenous fluorescent biomolecules and aldehyde fixatives on amino groups of lysine residues experimentally induced during sample preparation. Unfortunately, background autofluorescence is not absolutely eliminated by tissue clearing process [80]. Eliminating unwanted autofluorescence in the image is not automatically achieved during conventional tissue clearing processes expatiated in this review; therefore, the appropriate bleaching procedures amid sample preparation such as chemical agent treatment or

photo-irradiation, or spectral unmixing, a process of decomposing the spectral signature of mixed pixels into a group of relative abundances during image acquisition, should be fulfilled for hyaloid CLARITY tissue sample freed from autofluorescence [71, 81]. Introduction of CLARITY to the integrated laboratory workflow of histopathology for a clinical diagnosis with an abundance of visible evidences is now pending due to its compatibility with frozen section and formalin-fixed human specimens. Indeed, volumetric histology and 3D digital pathology are nowadays utilized in biomedical research due to its usefulness in reconstruction of topographical and spatial organization with structural framework accommodating specific patterns of physiological activity [82]. Three-dimensional techniques for CLARITY: tissue-gel hybridization, delipidation, fluorescence multi-labeling, RI matching and 3D confocal imaging with deconvolution also allows transparent volumetric image data followed by computational quantitative analysis for clear distinction of sporadically scattered opaque molecules along the entire tissue. There has been an attempt to apply the tissue clearing technique to histopathological diagnosis in human lung and lymph node [83]. Furthermore, conventional histopathological staining methods: hematoxylin and eosin (H&E), and immunohistochemistry are available after tissue clearing process for further study. Simultaneous process of both tissue clearing and routine conventional staining from one sample tissue in the laboratory probably gives much more options to medical laboratory scientists since the collective whole imaging data set expatiates every single story of sample tissue. Amelioration of several drawbacks of CLARITY also needs to be deeply considered: reduced exposure time to detergent, replacement of toxic clearing reagents, tangible hardness of tissue-gel hybridization for maintaining its original structure without swelling, shrinking and crumbling after tissue clearing and incremented antibody travelling efficiency for successful labeling with various fluorescent dyes. Taken

together, CLARITY is the appropriate equipment-based tissue clearing method which extends higher compatibility with routine laboratory workflow in clinical pathology where a vast sample of tissue are daily conducted in a time-effort-money effective manner and medical technologists engaged in mostly prefer the automation system for fixation, hydrogel polymerization and lipid extraction that is related to increased productivity and efficiency in processing multiple tissue samples obtained simultaneously from patients.

요약

조직병리학에서 현미경을 이용한 삼차원적 접근법은, 이차원 단면의 조직 슬라이드에서 박절 과정 중 부차적으로 발생하는 공간정보의 손실로 인하여 확인하기 어려웠던, 조직 내부 분자들의 공간적 배열, 상호결합, 구조적인 형태와 이들의 통합적인 공간적 정보체로서, 조직 내에 복잡하게 얽혀진 다양한 정보를 풀어내는데 있어서 복합적인 데이터를 제시하여 준다. 이광자 현미경(two-photon microscope)과 자동화된 보정환(correction collar)이 탑재된 고성능 대물렌즈의 개발과 같은 광학장비 영역의 발전은 조직투명화 과정을 거치지 않은 두꺼운 시료의 이미징에 있어서 광학적인 이론과 실제 사이에 존재하는 격차를 줄이는데 기여하였다고 할 수 있다. 하지만, 대물렌즈의 길어진 작동범위(working distance)와 최적화된 고강도 레이저의 사용으로 연계 되는 이점들은 세포 내 각 구성요소의 굴절률(refractive index) 차이로 인하여 증가되는 빛의 분산(light scattering) 현상으로 인해 자연스럽게 감소하게 된다. 조직투명화 기술이 처음 등장하였던 초창기 시도되던 간단한 굴절률 일치화(RI matching) 기법에서부터 현대의 최첨단 통합 조직투명화 기술에 이르기 까지를 관찰하여 볼 때, 형태학적인 변화 없이 조직의 투명도를 높이는 것과, 내재적으로 또는 고정과정 중에 유래되어 혼합된 자가형광 노이즈를 효과적으로 제거하는 것이 선명한 이미지를 얻기 위한 주요한 고려대상이라고 할 수 있다. CLARITY는 장비에 기반한 조직투명화 기법으로서 임상 조직병리 실험실에서 처리되는 동결절편과 포르말린에 고정된 검체 모두의 투명화를 위한 실험실 작업흐름(workflow) 통합 및 일상적인 실험절차와 호환이 가능할 것으로 보여진다.

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