Progress in ex situ tissue optical clearing – shifting immuno-oncology to the third dimension

Pawe Matryba, Leszek Kaczmarek, Jakub Gob

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Introduction
Tissue optical clearing (TOC) relies on the application of the selected optical clearing agents (OCAs) that enhance the optical properties of the studied tissue by either decreasing its light absorption and/or scattering coefficient. This, in turn, limits the number of interactions between photons and the sample, thus improving its penetration by laser light of the microscope setup [1]. The concept of TOC covers two distinct fields of research, namely in vivo [2, 3] and ex situ TOC [4], that currently rely on different OCAs and aims of their application. In vivo TOC utilizes OCAs that fall into three general categories of chemicals [5]: sugars (i.e., glucose, sucrose, fructose); alcohols (i.e., glycerol, sorbitol, propylene glycol); and electrolyte solutions that are widely used as X-ray contrast tose); alcohols (i.e., glycerol, sorbitol, propylene glycol); and electrolyte solutions that are widely used as X-ray contrast

Almost noninvasive diagnosis of potentially malignant tissue [10, 11]. Researchers interested in getting in-depth knowledge on in vivo TOC are highly recommended to refer to a recent comprehensive review on this topic by Oliveira and Tuchin [5], while we continue discussion on the ex situ TOC.

Ex situ TOC refers to all studies in which OCAs are applied on isolated and fixed organs. Incidentally, this type of tissue clearing is sometimes called “ex vivo” in the literature to underline the opposition to “in vivo” clearing; however this is misleading, as “ex vivo” implies studies on unfixed tissues/ organs to study how a particular physical/chemical factor affects the living tissue under conditions that are usually intolerable for a living organism. The main idea behind ex situ TOC is different from in vivo TOC, and aims to allow researchers to study tissues with resolution similar to the one offered by classical histology with a three-dimensional view, often spanning the entire organ of interest. Similarly to in vivo TOC, in ex situ TOC, one can readily distinguish different chemicals that this time can be classified into four basic groups [12], i.e., organic solvents, hyperhydrating solutions, high-refractive index aqueous solutions, and tissue transforming techniques. As reviewed recently [4], all these groups influence rodent organs in distinct ways, and thus possess either advantageous or disadvantageous features depending on the type of organ studied and biological question to be answered.

Here, we aim to succinctly highlight the most characteristic features of each ex situ TOC group; focus on current limitations
Overview of ex situ TOC methods

In the following section, a description of the major ex situ TOC approaches is provided, with their current taxonomy summarized in Figure 16.1.

Organic solvents

Historically, organic solvents were the first OCAs, proposed as early as 1911 by Spalteholz [13]. Rediscovered 100 years later in the 2010s, these agents are currently widely used due to the exceptionally high refractive index (RI) easily achieved by these solutions. Such high RI (1.54–1.56) matches that of proteins (>1.50) and, in general, guarantees the highest transparency of the resultant sample [14]. It should be underlined, however, that the light transmittance measured for brains cleared with organic solvents is the highest vs. other clearing methods starting from 550–600 nm [15, 16], as these possess an amber-like color that limits transmittance in shorter wavelengths. This might explain why multiplex detection is rather avoided with organic solvents, with dyes usually excited at ~650 nm (recently in the field of TOC, application of dyes that are excited in the near-infrared region has also emerged [17]). However, excitation in the spectrum of green fluorescent protein (GFP) is not useless, as it detects autofluorescent signals that can help position the target of interest within the context of the tissue or even serve as a reference for Allen Brain Atlas annotation in the case of this organ [18].

Rapid decay of the fluorescent signals of proteinaceous fluorophores and toxicity were perceived as major weaknesses of organic solvents, with both of these being addressed in the following protocols [19]. As GFP/YFP (YFP – yellow fluorescent protein/RFP – red fluorescent protein), etc. (hereafter abbreviated as XFP for simplicity, if applicable) were suspected to require water molecules to sustain their function [1], it was initially believed that quenching of proteinaceous fluorophores would remain an inherent limitation of organic solvents that, immiscible in water, need the tissue to be completely dehydrated before the RI matching step. This belief, however, was proved misleading by several research groups that independently presented that fluorescence might be greatly stabilized simply by using slightly alkaline (pH 9.0–9.5) chemicals. This was validated for both dehydrating agents (tert-butanol in the case of FluoClearBABB [20], a-uDISCO [21], and PEGASOS [22], THF in FDISCO [15], ethanol in ECI [23], and 1-propa-nol in 2nd generation of ECI [24]) and RI matching organic solvents (BABB-D in a-uDISCO [21]). Besides alkalization, the introduction of peroxide scavengers to both dehydrating alcohols and RI matching organic solvents might be beneficial for XFP preservation. Apparently, the first such attempt was presented by Pan et al. [25], who added alpha-tocopherol to the final RI matching solution, and this approach has recently been exploited by Hahn et al. [26]. In this study led by Dodt, a pioneer who combined organic solvent-based TOC and ultra-microscopy [27], researchers showed that these are both aldehydes and peroxides that contribute to rapid quenching of XFP fluorescent signals. Thus, their protocol relies first on the elimination of aldehydes and peroxides by column chromatography with basic activated aluminum oxide from THF and DBE (dehydration and RI-matching solution, respectively). Second, as peroxides tend to develop with time and contribute to the
generation of new aldehydes, a set of promising compounds that are known to prevent peroxide generation was tested, revealing propyl gallate to be the most effective inhibitor of peroxides from DBE (significantly more effective than, e.g., alpha-tocopherol). Importantly, the resultant protocol, named sDISCO, greatly stabilizes the XFP signal, so that successful imaging can be performed 1 year after the clearing (and possibly later, but no further data regarding stability was provided).

Notably, all of the steps of the animal perfusion, fixation, and TOC were performed at 4°C (as in FDISCO protocol) and with slightly alkaline PBS (pH 8.3), both of which conditions were reported to stabilize signal from XFPs.

In order to overcome the toxicity of organic solvents serving as RI-matching solutions, which are dangerous for both human health (potential carcinogens) and a vast majority of standard microscopic lenses and other parts of the imaging apparatus, ethyl cinnamate (ECi, approved food flavor) was introduced by Klingberg et al. [23] to first study kidney, bone, and heart, the application of which was further extended by Masselink et al. [24] on such distinct samples as *D. melanogaster*, *X. laevis*, and cerebral organoids, as well as adult and larvae of axolotl.

To summarize, the main drawbacks of organic solvent-based TOC, i.e. preservation of XFPs signals and toxicity, have already been overcome, leaving researchers with an easy-to-apply, rapid, cost-effective, and efficient TOC method. However, solvent-based TOC is not yet "one size fits all," and success with applying this set of methodology still depends on reflection on three additional factors: 1) the type of endogenous chromophores and/or light-absorbing molecules, 2) compatibility with immunohistochemistry and lipophilic stains and 3) effective resolution required to accurately answer the biological question.

Endogenous chromophores such as heme, melanin, lipofuscin, and riboflavin absorb light in its visible spectrum and as such, might severely limit the quality of the acquired data [28]. Although pioneer solvent-based TOC methods, like a BABB/Murray’s clear, could not eliminate these light-absorbing molecules, novel ideas and progress in other chemical groups of TOC have now allowed researchers to profit from both the remarkable transparency achievable with organic solvents and the removal of endogenous chromophores (mainly the most abundant heme – present in both hemoglobin and myoglobin proteins). This obstacle might be overcome either by the introduction of a bleaching step or heme elution. A bleaching step with 5–10× diluted 30% H₂O₂ in methanol was first introduced in the widely utilized iDISCO protocol [29] and later optimized with the addition of sucrose specifically for clearing and decolorization of mouse immune organs (the spleen is exceptionally difficult to decolorize, followed by subcutaneous and mesenteric lymph nodes and thymus) and presented under the name ImmuView [30]. It should be noted, however, that the harsh treatment of tissue during iDISCO might lead to the loss of antigenicity of particular epitopes and/or reactivity of clones of antibodies, i.e. CD31 [31, 32], which therefore must be carefully screened during preliminary experiments. A constantly updated list of already validated antibodies can be found at https://idisco.info/validates-antibodies/. Decolorization with Quadrol (N,N′,N′′-tetrakis(2-hydroxypropyl)ethylenediamine), a heme-releasing aminoalcohol [33], was first reported in CUBIC (one of the pioneer methods from the hyperhydrating solutions group) and later incorporated as additional step before dehydration in case of solvent-based TOC [17, 22]. Notably, such an application has so far been reserved for studies employing so-called whole-body clearing (which aim at making transparent the majority of, if not all, rodent organs) to ensure efficient penetration of organs by Quadrol during the perfusion step.

The other issue worth briefly mentioning is the possible incompatibility of immunohistochemistry with organic solvents even without harsh methanol/H₂O₂ pretreatment [34, 35]. Although this problem might be attributed to any group of TOC methods, loss of antigenicity seems to be more pronounced in case of solvents i.e., 3DISCO [34, 35]. However, very recent articles also show that a combination of, first, tissue incubation in CUBIC, followed by a solvent-based TOC, might significantly enhance immunostaining efficacy either by decreasing background signals or enhancing antibody penetration [36–39].

Furthermore, the excellent transparency of these protocols comes at the expense of, at least partially, lipid solvation which makes them incompatible with lipophilic stains that are widely utilized for neuronal and vascular tracing, for example DiI [40]. Last but not least, the dehydration step causes anticipated tissue shrinkage of the specimen that ranges from ~10% in case of skin to as much as ~35% shrinkage of brain tissue [41]. This might be especially advantageous when a large volume of tissues, even whole bodies, need to be screened, as elegantly presented by Pan et al. [42], who applied solvent-based TOC to quantitively approach dynamics of cancer metastasis using various cancer models. Moreover, by applying deep-learning algorithms, this team revealed the efficacy of antibody-drug targeting of metastases in the entire body.

**Hyperhydrating solutions**

As tissues are composed mainly of water (RI = 1.33) and proteins (RI > ~1.50), this natural RI mismatch can be overcome either by removing water (as in the case of organic solvent TOC) or tissue hyperhydration. Hyperhydration homogenizes tissue composition by decreasing its overall RI and partial dilution of light absorbers/scatterers. Hyperhydration solution TOC are represented by i.e. Scale [43], CUBIC [44], UbasM [40] (which utilizes a high concentration of mild detergents, such as Triton X-100) and Clear²/Clear² [45], RTF [46], and FRUIT [47] (in which detergents are almost absent). In general, the first group is capable of clearing large tissue blocks, organs, and even whole rodent bodies, while detergent-free solutions are much more widely applied to small samples such as spheroids [48] or embryos [46] at early stages of development. Undoubtedly, the most frequently applied, out of hyperhydrating solutions, is CUBIC, so far successfully used to study almost every murine [44] and rat [49] organ. What makes CUBIC protocols so widely utilized are two easy-to-prepare, inexpensive, and nontoxic solutions (designated as CUBIC-R1 and -R2), which, thanks to the already mentioned Quadrol, also effectively decolorize the organs. Although in principle CUBIC-R1 was developed to perform delipidation (15 wt% Triton X-100), decolorization (25 wt% Quadrol), and hyperhydration (25 wt% urea), with
CUBIC-R2 serving as high RI solution for RI-matching (50 wt% sucrose), we have recently presented that in the case of murine lymph node clearing, immersion in solely CUBIC-R1 is sufficient to perform successful, high resolution light-sheet imaging [35]. Based on the available literature, treatment with CUBIC-R1 rather preserves antigenicity of the majority of epitopes and allows for deeper antibody penetration due to “loosening” of tissue structure and its partial delipidation. Although images of traceable structures, such as neuronal projections or vasculature, are usually acquired with success, CUBIC-R1 can lead to distortion or even complete loss of signal from immune cell markers (e.g. CD4, CD8, B220) [34, 35]. Importantly, concentration of the paraformaldehyde (PFA) used during the fixation step can exert tremendous influence on the quality of the final image following immunohistochemistry, with 4% PFA preserving immune cell markers more efficiently than 1% PFA, which further confirms the possible negative impact of CUBIC-R1 on tissue protein content or at least their structure [35] (Figure 16.2). Thus, if the quality of the final image seems to be compromised, it is advised to perform immunolabeling before CUBIC TOC, as long as the penetration of the full tissue thickness can be achieved.

Further imperfections of CUBIC protocols are their incompatibility with DiI labeling [40] (observed again due to high concentration of Triton X-100 but overcame in UbasM technique) and moderate stabilization of XFPs fluorescence. It is important to note that while CUBIC properly stabilizes XFPs fluorescence and quenching of XFPs to the level at which these are undetectable is improbable [50], samples left in CUBIC-R1 will gradually decrease signal intensity over time [35, 50]. Thus, it is pivotal to perform imaging during the same time points after the clearing, if a fluorescence intensity is measured for quantification.

Moreover, the original CUBIC solutions could not transparentize bones [51] (making actual whole-body imaging inaccessible), and Triton X-100 was not an ideal delipidating agent. Thus, the recent efforts of Tainaka et al. [52] led to the development of “second generation” CUBIC solutions that are now capable of more efficient delipidation, decolorization, decalcification, and RI-matching, allowing actual whole-body clearing to be performed for the first time.

Application of new delipidating (10 w%/10 w% N-butyldiethanolamine/Triton X-100, aka CUBIC-L) and RI-matching (45 w%/30 w% antipyrine/nicotinamide) solutions allowed the same research group to study whole-body/organ cancer metastases in a number of widely used murine models (Panc-1, SUIT-2, B16F10, to name just a few), with spectacular resolution, allowing for detection down to a level

![FIGURE 16.2 Efficiency of immunostaining performed on CUBIC-cleared samples might be compromised. CUBIC was already successfully applied in >150 studies, but some of them report compromised retention of protein content [77], which might lead to a poor/absent immunostaining signal [34]. We have recently found that if the precise morphology of cells needs to be retained for further analysis, immunostaining performed before clearing might be beneficial in the case of particular epitopes [35]. Moreover, fixation of tissue with 4% PFA, when compared with 1% PFA (regularly used during fixation of immune organs), may further increase the quality of the resultant image. Postclearing, immunostaining was performed after TOC; preclearing, immunostaining was performed before TOC. Scale bar, 50 mm. Reproduced under AAI guidelines from reference [35].](image-url)
of a single cancer cell. Further increase in delipidation efficacy was presented by Inoue et al. [53], who introduced 1,2-hexanediol as a potent agent for blocks of lipid-rich human white matter specimens.

Perhaps the most prominent limitation of the current CUBIC pipeline is still a relatively long processing time. Although hands-on time is short, the whole-body clearing can take up to 2–3 weeks in case of adult animals. This was very recently overcome by Zhu et al. [54], who introduced a new TOC solution that is based on m-xylendiamine (MDXA). Analogously to urea, MDXA presents hyperhydrating capability due to the presence of two NH\textsubscript{2} groups; however, its water solution achieves a much higher RI (up to RI = 1.57), similar to that of organic solvents. The group prepared three solutions which gradually increased concentration of MDXA and sorbitol, termed MACS-R0, -R1, and -R2 (MDXA-based Aqueous Clearing System), which first assure that the entire organ/organism is well penetrated with diluted solution (R0) and later homogenize tissue RI (R1) and finally match RI for the imaging (R2, RI = 1.51, as the final solution guarantees almost intact tissue size, MACS should be perceived as a high-refractive index organic (MDXA) and sugar (sorbitol) aqueous solution described in detail in the next section). Such high RI of the final of MACS solutions efficiently transparentize the entire body of adult mouse, even bones, without the need to perform prior decalcification and thus opens the possibility for further utilization for whole-body imaging. Moreover, it was observed that MACS decolorizes heme-rich tissues, possibly in a mechanism of Fe release. A number of the performed quantifications confirmed that MACS is a rapid TOC protocol (rendering mouse brain transparent in as little as 2.5 days), entirely compatible with lipophilic dyes, a good stabilizer of XFPs fluorescence (e.g. EYFP, EGFP, tdTomato), and an efficient tissue-decolorizing agent applicable to both embryos and whole organs/organism. However, it also relies on toxic reagent, as MDXA is fatal if inhaled (category 2), labeled as toxic and corrosive by GHS, and assigned to health hazard category 4 by NFPA and 3 by HIMS, (meaning “deadly” and “serious hazard”, respectively).

High-refractive index aqueous solutions

Although vastly heterogenous from a chemical perspective, the protocols that fall into this TOC group, which consist of one- to two solutions or gradient solutions of sugar, are mainly applicable to small pieces of tissue (e.g. 1 mm-thick brain slices, lymph nodes, organoids), and achieve final RI of ~1.46–1.50. This group of reagents is represented by sucrose and glycerol solutions, SeeDB [55], SeeDB2 [56], FRUIT [47] (being at the border of hyperhydrating solutions due to urea and high-RI aqueous solutions due to gradient of fructose), second solution of UbosM [40], FocusClear\textsuperscript{TM} [57], 2-2’ thiodiethanol [58], C\textsubscript{3}D [34] (clearing-enhanced 3D microscopy), the recently presented FOCM [59] and FUNGI [60, 61], and the already described MACS [54].

Gradients of sucrose and fructose (SeeDB) were the first to reveal the potential of high-RI aqueous solutions to render mouse brain transparent. Although SeeDB is a poor TOC method for whole-organ clearing, it is still successfully applied to a variety of thick tissue sections imaged with confocal microscopy [62, 63]. The second generation of SeeDB, SeeDB2, was also invented for imaging brain tissue sections, but this time with superresolution (achieving lateral resolution of 50–150 nm). Utilization of a patent-protected FocusClear was at first vast, ranging from mammals [57] and insects [64] to plants [65] and even biomaterials [66], and serving as mounting medium for CLARITY-cleared samples, but it is now limited by its relatively high price. Similarly to CUBIC, UbosM protocol relies on two solutions made of urea, with additional chemicals introduced to prevent tissue swelling and to replace a high concentration of Triton X-100, which (in comparison to CUBIC) makes UbosM-treated samples compatible with Di labeling. Recently, a simple incubation in 75% and 88% v/v glycerol solutions were shown to be highly efficient for both spheroids [67] and murine lymph nodes [35], respectively. Unfortunately, long-term storage of lymph nodes in 75% glycerol leads to tissue deformation and quenching of XFPs, EGFP, and DsRed in particular [35, 68].

Recent efforts in the development of high-RI aqueous solutions aimed to generate protocols that would render samples transparent rapidly and preserve their endogenous fluorescence. These criteria are fulfilled by two chemically similar methods – FUNGI [61] and FOCM [59] – and distinct from them, the C\textsubscript{3}D method. FUNGI, consisting of 50% v/v glycerol, 2.5 M fructose, 2.5 M urea, 10.6 mM Tris Base, and 1 mM EDTA, was successfully applied to a number of organoids and small murine organs, rendering them transparent within as little as 2–4 hours. Notably, FUNGI-cleared samples can be stored in this reagent at ~20°C for at least 18 months and thawed without any significant loss of fluorescent signal. On the other hand, FOCM is made of urea, d-sorbitol, and glycerol dissolved in DMSO, the ratio of which depends on the size of the organ to be cleared, and it was to be proved useful for TOC of both hemisphere and thick brain slices. In case of the latter, FOCM achieves spectacular clearing time, requiring as little as 2 minutes to render a 300-\textmu m-thick slice transparent and ready for confocal imaging. Similarly to FUNGI, FOCM neither quenches XFPs nor causes tissue distortion. C\textsubscript{3}D [34] is another, very simple, “one-pot” TOC method that relies on N-methylacetamide and Histodenz and guarantees robust tissue transmittance of a variety of murine organs. Excellent retention of both fluorescence of endogenous XFPs and the epi-topes, as confirmed by multiplex immunohistochemistry, with rapid clearing taking 1 day to complete and the possibility of detecting RNA in clarified samples [69], makes C\textsubscript{3}D a promising candidate for widely utilized TOC method. However, two possible limitations of C\textsubscript{3}D exist: inability of tissue decolorization and minor (10%–20%) shrinkage of tissue volume [34, 35, 70] that might impede object segmentation, especially in case of densely packed immune cells.

Tissue transforming methods

The CLARITY protocol [71] (Clear Lipid-exchanged Acrylamide-hybridized Rigid Imaging/Immunostaining/In situ hybridization-compatible Tissue-hHydrogel), in which acrylamide/bisacrylamide solution creates a hydrogel mesh inside the tissue, laid the foundation for a new branch of
methods that transform tissues into rigid tissue–hydrogel matrices. Since the first report of relatively complex protocol of CLARITY in 2013, which relied on electrophoretic tissue clearing for lipid extraction with a strong detergent, as well as the performance of sodium dodecyl sulfate at high temperatures, which often led to tissue damage, a plethora of advanced CLARITY protocols and modifications have been developed [72, 73]. These modifications include titration of monomer solutions, their concentration [74] and composition [75, 76], resigning from electrophoretic delipidation [77]; the development of new, stable devices for stochastic electrotransport [78] to improve both clearing and labeling efficiency; and the introduction of new, inexpensive RI matching solutions [75, 77], with new polymers still being designed and tested for possibly better mechanistic properties for the TOC process [79].

Currently, apparently the most advanced techniques in the field of tissue transformation come from Chung’s and Boyden’s laboratories. A novel tissue preservation method called SHIELD [80] (stabilization to harsh conditions via intramolecular epoxy linkages to prevent degradation) utilizes polyglycerol 3-polyglycidyl ether as a resin acting in a tissue as a cross-linker that forms additional intramolecular bonds and protects tertiary structure of proteins. Briefly, a solution that contains polyglycerol 3-polyglycidyl ether is first infused along with PFA during the perfusion step of the animal; next, selected organs are additionally immersed in a similar solution but one devoid of PFA, and finally, a process of crosslinking is initiated at 37°C. SHIELD ensures excellent preservation of tissue structure upon TOC, antigenicity (with more than 50 antibodies validated in original article), and fluorescence of XFPs even under harsh conditions e.g. 24 hours of incubation at 70°C. Another issue optimized by Chung’s group is the uniform immunolabeling of large organs. SWITCH [81] was the first protocol developed for that purpose that relied on two solutions – SWITCH-off, which inhibits binding of antibodies with the tissue, thus allowing for their unlimited spread, followed by SWITCH-on, which restores the tissue’s and the antibodies’ reactivity. Unfortunately, one should be bear in mind that the process of tissue immersion with SWITCH-on also takes time, which means that there is a gradient of tissue-probe reactivity from the border to the core of the specimen, potentially still leading to uneven labeling. The most recent idea to overcome the issue of uneven immunohistochemistry of large specimens, such as mouse brain hemisphere, was presented in a protocol named eFLASH [82] (electrophoretically driven fast labeling using affinity sweeping in hydrogel). The idea behind eFLASH is to gradually increase the affinity between probes and their targets by balancing them with a concentration of bile salts, e.g. sodium deoxycholate, and pH. Yun et al. [82] observed that the affinity of various antibodies rises as the concentration of bile salts decreases and pH falls (from basic to neutral). Thus, since the start of the protocol, the probes can penetrate fixed and delipidated samples in an unbiased manner without the risk of a huge depletion of antibodies at the sample’s border. Such a protocol not only provides an opportunity to perform unbiased immunolabeling of large tissue volumes, but also greatly decreases the amount of probes needed, which are otherwise significantly depleted before reaching the core of the specimen (if their targets are highly abundant in a particular tissue). When tested on a SHIELD-fixed, delipidated tissues with immunolabeling performed using the previously described technique of the stochastic electrotransport, eFLASH collectively allowed for the uniform labeling of murine brain with as little as 3–5 μg of antibody, depending on the target. Boyden’s group renders tissues transparent via intensive expansion of the transformed samples [83]. Development of protocols that rely on gel anchoring, followed by digestion of proteins and immersion (expansion) in water, results in 4.5x linear tissue expansion (or even ≈16–22x, if the process of gel anchoring and expansion is repeated) and >99% composition of the resultant tissue of water. However, it should be noted that the final tissue transparency is rather a side-product, as this team aims at superresolution microscopy, e.g. capturing the morphology of dendritic spines with conventional spinning disk systems, rather than at imaging exceptionally large volumes of optically cleared specimens.

To summarize, tissue transforming techniques offer plenty of novel methodological advances that make such TOC convenient, but could be implemented with other groups of TOC to strengthen the applicability of the latter. Although fixation with the SHIELD methodology seems advantageous for the majority of TOC techniques, its application is rather limited, perhaps due to the doubts of researchers regarding work with potentially toxic resins. The commercialization of easy to use setups for SHIELD, stochastic electrotransport, or eFLASH could possibly widen implementation of these tools in research groups composed mainly of biologists.

### Application of TOC to immune organs

Although TOC has already been successfully applied to literally every murine organ, the immune organs appear to be extraordinarily challenging to clear and image. This is either because of a high abundance of hemoglobin (i.e. spleen and bone marrow), or extremely high cellularity (i.e. lymph nodes), which hinders quantitative measurements. Here, applications of TOC to primary and secondary immune organs, along with their limitations and perspectives, are highlighted.

#### Bone and bone marrow

Having less dense distribution of cells, as compared with other lymphoid organs, bone marrow (BM) offers favorable whole-organ imaging and segmentation conditions. Due to heterogeneity of bone tissue (hard, solid minerals and soft marrow [84]) and relatively high RI [85] of its components (e.g. apatite ~1.62 [86], lipids ~1.45 [87] and collagen type I ~1.43 [88]), initially only organic solvents could effectively match the RI of bones. One of the first applications of TOC, to half bone and BM plugs, was presented by Acar et al. [89], who, by using Murray’s clear (dehydration in methanol followed by RI-matching in BABB), visualized possible distinct niches in BM that are occupied by populations of either dividing or nondividing hematopoietic stem cells (HSCs). During the selection of an appropriate TOC approach, the authors observed that harsh, SDS-mediated lipid
removal during either electrophoretic (CLARITY) or passive (PACT) protocols did not offer optimal clearing conditions and, moreover, resulted in the destruction of several cell surface epitopes. Although expected, it was also experimentally proven that other TOC methods that are characterized by rather low RI (e.g. CUBIC, Focus Clear, and ScaleA2) were not effective for bone/BM clearing. Finally, besides Murray’s clear, the 3DISCO technique (dehydration with THF followed by RI-matching in dibenzyl ether) was applied by Acar et al. [89] during experiments in which XFPs were visualized, as it led to better stabilization of DsRed and tdTomato signal, but resulted in worse clearing efficiency otherwise. Similar results were obtained by Berke et al. [90], who performed a side-by-side comparison of eight TOC methods. In addition, this group showed that it is not only the value of RI that influences the efficacy of bone TOC, but also the mechanism of clearing (i.e. dehydration vs. hyperhydration) as 97% TDE (RI ~1.47) outperformed other high-RI solutions, such as SeeDB (RI ~1.50). The detailed protocol for solvent-based TOC, bone/BM imaging, and segmentation was recently described by Gorelashvili et al. [91]. Using this pipeline and in vivo two-photon imaging, the authors could provide strong evidence for a revised model of megakaryopoiesis, in which megakaryocytes are located at the BM sinusoids and are being replenished by the precursory cells that originate not from the periostic but rather from the sinusoidal niche [92]. Using an ethanol-ECl protocol (called “simpleCLEAR”), Grünbaum et al. [93] discovered a new type of vessels that originate in BM, travel perpendicularly through the cortical bone, and finally connect to periosteal circulation. These structures, named transcortical vessels (TCVs), are highly abundant in both murine and human bones, and express either arterial or venous markers. Interestingly, this group found TCVs to exhibit significant remodeling capability, i.e., new TCVs develop within weeks in a model of chronic (but not acute) arthritis (thus potentially contributing to enhanced efflux of leukocytes to joints) and decrease in number upon irradiation and aging. A variation of another solvent-based TOC, iDISCO, was recently presented as the BoneClear [94] method. The major novelty introduced in BoneClear is to combine the iDISCO scaffold with prior bone decalcification with EDTA. BoneClear-processed murine bones exhibit unprecedented transparency and excellent immunostaining (as verified with nine primary antibodies) and XFP preservation capabilities. As a proof of concept, the authors applied BoneClear and visualized the entire innervation of femur and hindpaw, followed by the process of re-innervation upon injury and neuropathy in chemotherapy-induced murine model (treated with Paclitaxel).

It should be underlined, however, that the successful application of organic solvents to bone/BM imaging seems to heavily rely on the experience of a particular research group. As already noted, Acar et al. [89] reported that 3DISCO stabilized the signal from XFPs better than Murray’s clear, while Berke et al. [90] has shown that it is 3DISCO that leads to the most dramatic loss of XFPs when compared to other solvents. Moreover, Coutu et al. [95] screened more than ten clearing-mounting media specifically for the purpose of BM imaging and reported “efficient bone and marrow clearing” with ethanol-ECl, but also “high background fluorescence and staining artefacts, massive tissue distortion, and shrinkage,” while the same protocol in the hands of the Gunzer group [93] allowed them to obtain compelling imaging conditions and discover, as mentioned above, CTVs. As the advanced protocols from all other chemical groups of TOC already achieve optimal and similar bone clearing (i.e. Bone CLARITY [96], a modified CLARITY approach, in which an additional step of EDTA-mediated decalcification and decolorization with Quadrol was introduced, and CUBIC-B [52], which relies on EDTA and imidazole cocktail for the efficient decalcification), it is now even more difficult to select the best method [97]. Most probably, such a method simply does not exist, and the choice should be made upon initial screening to verify suitability with particular bones/epitopes/XFPs. Nonetheless, both Bone CLARITY and CUBIC were already applied to bone clearing (besides the proof of concept studies performed by the inventors) and facilitated description of the dynamics of HIV-1-mediated spread and infection of human CD4- and CD68-expressing macrophages in humanized mice [98] and near homogenous attachment of long-term HSCs to the vascular endothelial cadherin positive cells [99], respectively. Undoubtedly, progress not only in TOC approaches but also in new light-sheet microscopy setups will greatly add to high-throughput and precise imaging of bone and BM [100].

TOC of the isolated BM seems to be relatively easier than that of the whole bone [101]. The first such technique was implemented in a study published as early as 2013, in which Nombela-Arrieta et al. [102] applied FocusClear to visualize distribution of hematopoietic stem and progenitor cells in the BM of murine femoral bones. Interestingly, they found that although these cells are preferentially located in endosteal zones, in close contact with the microvessels, they exhibit a strong hypoxic phenotype defined by e.g. expression of HIF-1α. The same group has recently presented optimized multiscale 3D quantitative microscopy of BM cellular components using RapiClear (RI ~1.52) as an OCA [103]. The experimental workflow, in which BMs are imaged with confocal microscopy, first using low magnification (10–20×) to define regions of interest and then higher magnification (40–95×) to achieve subcellular resolution, results in a mosaic image that is finally converted into single volumetric reconstruction, as presented in Figure 16.3. Using such an approach and flow cytometry, Gomariz et al. [103] found tremendous discrepancies between these methods, when stromal cells within the entire murine femurs were to be counted. 3D quantitative microscopy revealed ~30x greater numbers of both sinusoidal endothelial cells and CXCL12-abundant reticular cells, a fundamental knowledge which greatly supports the need for the introduction of histocytometry approaches for the reevaluation of cellular composition of tissues. A similar histocytometric approach to BM imaging was presented by Coutu et al. [95], who selected TDE as an optimal TOC chemical. In addition to the TOC pipeline, the authors developed the software x-dimensional image analysis toolbox (XiT) for high-throughput image analysis, which allowed them to elucidate relationships between hematopoietic cells, bone matrix, and marrow Schwann cells.
Spleen

Although extremely rich in light-absorbing pigments, especially heme, spleen has already been optically cleared by a number of laboratories, using every chemically distinct TOC group [22, 44, 74, 75, 104, 105]. It should be noted, however, that in the majority of cases, only macrophotographies were provided without fluorescent imaging to assess whether the residual pigments do not compromise usefulness of the particular TOC method [22, 40, 41, 75, 106]. Thus far, application of BABB as a RI-matching solution to thick spleen segments has facilitated precise annotation of HSC localization and the discovery of new migratory capacity of neutrophils [107]. Using ImmuView (an iDISCO-based protocol, the chemical composition of which has already been discussed here), Ding et al. [30] presented a compelling characterization of the sympathetic and parasympathetic innervations of the spleen and other major immune organs (subcutaneous and mesenteric lymph nodes, Peyer’s patches, thymus) and proved >99% of synaptophysin positive (pan-neural marker) neural fibers to be of the sympathetic (TH positive) type (Figure 16.4). 3D visualization of spleen neuroanatomy was also assessed by Murray et al. [108], who, by applying the CLARITY protocol, quantified interactions between TH-positive axon endings and populations of choline acetyltransferase expressing CD3+ T- and B220+ B-cells. Lastly, CUBIC was successfully applied to the spleen by Kieffer et al. [109] to aid tracking of HIV-1 and HIV-1 infected CD3+ T-cells spread, similarly to the already outlined, bone-oriented experiment [98].

Lymph nodes

Similarly to spleen and BM, the possibility of visualizing LNs in 3D was appreciated by several research groups that had already utilized every chemical group of TOC methods for that purpose [68, 69, 110, 111]. Although small and relatively pigment-free, successful imaging of LNs characterized by immense cellular density still requires the prudent selection of TOC. We have recently performed a profound evaluation of >10 protocols that span the entire chemical spectrum of TOC methods [35]. After side-by-side comparison of the resultant LN transparency, size change, compatibility with proteinaceous fluorophores (GFP and RFP, in particular), immunostaining, and H&E staining, it should be concluded that both CUBIC-R1 (first reagent of classical CUBIC protocol) and C3D guarantee optimal TOC conditions for murine LNs. Nevertheless, one of the first studies in which murine LNs were subjected to solvent-based TOC was presented by Woodruff et al. [112] who, by using a model of influenza vaccination, revealed rapid repositioning of LN-resident dendritic cells (DCs) from the T-cell cortex to the medullary interfollicular region. Interestingly, the resident DCs captured virus effectively (represented by expression of CXCL10) and activated viral-specific CD4+ T-cells (represented by expression of CD69, CD44, and CXCR3) even before the expected time of arrival of migratory DCs from skin. BABB-mediated RI-matching was also a core technique utilized by Kumar et al. [113, 114] to study lymphoid remodeling upon viral infection with special emphasis on number and volume of B-cell follicles and length of high endothelial venules (HEV). The dynamics of tumor-induced lymphangiogenesis in 4T1 (breast cancer) and B16F10 (melanoma) murine models was also genuinely revealed by Commerford et al. [115] who used BABB clearing. Although these studies were very informative, it should be reemphasized that dehydration observed in case of solvent-based TOC always leads to severe tissue shrinkage (even 50%–60% of original LN area), thus precluding further direct quotation of such results to define e.g. total length of HEV network under physiological conditions, as unfortunately still is the case [116]. Moreover, tissue shrinkage is perceived to be advantageous when large tissue volumes need to be imaged and the targets of imaging are positioned relatively loosely, i.e. neuronal projections, vasculature, or sites of metastasis. Thus, in a majority of studies trying to quantitatively elucidate immune responses in 3D fashion by inspecting changes in numbers of immune cells, organic solvents will significantly hinder analysis. This issue was already reported by Cabeza-Cabrero et al. [117] who, after applying uDISCO to mesenteric LNs and spleen, were unable to study distribution and clustering of DCs in this organs, and thus limited their analysis to nonlymphoid tissue, lung, and small intestine in...
particular. Meager but consistent tissue shrinkage (between 25% and 10% volume reduction depending on organ, with lung and bone retaining their original size), seems to be also the only weakness of an otherwise excellent Ce3D method. Ce3D, applicable to the number of organs but developed with a view to enhance the histocytometry approach, was already applied to generate high-quality imaging data and develop advanced MATLAB toolbox for spatial cellular analysis, and called Histo-Cytometric Multidimensional Analysis Pipeline [118] (CytoMAP, Figure 16.5).

Another possible challenge that arises during imaging of the cleared LNs is the opacity of the surrounding adipose tissue, which is not cleared effectively with majority of TOC protocols (with the exception of organic solvents) and autofluorescent signal coming from the fibrous capsule (especially in the ~488 nm channel). The first issue should be addressed after LN fixation by gentle removal of the surrounding tissue, preferably under the stereomicroscope. The second limitation can be overcome by slicing LN into thick (200–500 μm) slices and starting the z-stack from the cutting plane. Thus, it is important to first decide whether true whole-mount imaging is indeed indispensable for answering a particular hypothesis. Imaging LN sections is definitely a more straightforward approach as these usually do not require power ramping nor do they present with a significant autofluorescent signal of the fibrous capsule. In addition, it greatly expedites immunostaining and overcomes the potential problem of uneven labeling. Studies performed with TOC applied to thick LN slices have already contributed to a number of discoveries of particular importance [110, 119]. For instance, Mondor et al. [120] presented a detailed scenario of LN vascular network remodeling in which it is orchestrated by the clonal proliferation of HEVs, while Dubey et al. [119] deciphered crosstalk between fibroblastic reticular and B-cells that promotes both de novo follicle formation and lymphangiogenesis.

**TOC in cancer research**

The possibility of tracing vasculature over long distances and performing fast inspection of entire organs or even murine bodies with cellular resolution prompted researchers to apply TOC in cancer research both at basic science and clinical levels. Although as early as in 2006, a case report by Dickie et al. [121] presented that highly detailed imaging of cleared, carbon perfused arteries of orthotopically implanted intracranial tumor is feasible with Murray’s clear, the first advanced study deciphering intratumoral vasculature comes from Dobosz et al. [122]. Using HER-2 overexpressing human breast cancer cell line (KPL-4) to generate tumor xenograft, injection
of lectin-Alexa Fluor 647, and BABB-mediated TOC, the researchers obtained a precise view of tumor vasculature and intratumoral distribution of Alexa Fluor 750-trastuzumab (a humanized monoclonal antibody that has a high affinity for the extracellular domain of HER2). While the distribution of trastuzumab was mosaic, the observed heterogeneity relied vastly on the composition of tumor tissue (with necrotic areas marginally penetrated when compared with soft, highly vascularized tumor compartments). Hence, the developed pipeline was employed to study the effect of administration of bevacizumab (humanized monoclonal antibody inhibiting growth of blood vessels) on trastuzumab distribution. Strikingly, intratumoral vasculature (number of vessel segments, branching points, vessel volume) was already significantly diminished 1 day after injection and strengthened over the entire period of treatment. This effect, however, was observed only in the case of vessels located at the tumor periphery and on vessels with a diameter between 10 and 30 μm and significantly decreased penetration of the HER2-targeting antibody. In a similarly designed study, Pöschinger et al. [123] applied TOC to verify the sensitivity of the dynamic contrast-enhanced microcomputed tomography as a tool for noninvasive assessment of efficacy of antiangiogenic compounds. Interestingly, the data obtained with subcellular resolution in case of TOC and light-sheet imaging was highly correlated by the results of in vivo micro-computed tomography. Relation between tumor and vasculature was also studied by Lagerweij et al. [32] in an orthotopic glioblastoma xenograft models. Using CLARITY and iDISCO on 3–5 mm-thick brain slices (to assure unbiased penetration of the antibodies) they could visualize migration patterns of glioblastoma tumor cells in relation to microvasculature. It turned out that in more vascularized, gray matter, tumor cells migrate in proximity of vessels, while being distant from vasculature in white matter, observation of which suggests migration behavior through tracts. The value of 3D imaging of glioblastoma was also presented by Yang et al. [124] who provided precise characterization of its microenvironment in terms of cell stemness, immune infiltration, and microvasculature. Angiotropism in brain metastases of skin melanoma was also recently presented by Rodewald et al. [125] in 3 mm-thick biopsies of eight patients, which were successfully cleared with CLARITY protocol. Cell migration of

![Scheme representing CytoMAP pipeline.](image-url)

**FIGURE 16.5** Scheme representing CytoMAP pipeline. (A) CytoMAP is a tool for highly accurate quantitative analyses to reveal how local cell microenvironments form global tissue structure and to study both intra- and intersample tissue heterogeneity. (B) After acquisition of high-resolution data, hierarchical gating of cell objects is performed and passed into CytoMAP for segmentation using clustering algorithms that ultimately allows exploration of detected clusters in 2D or 3D space. (C) In CytoMAP, several tools for data quantification were included, e.g. analysis of spatial correlations between different cell types, investigation of distance relationships of cells with architectural landmarks, analysis of neighborhood heterogeneity within individual tissues or across multiple samples, and quantitative visualization of tissue architecture. Adapted under the terms of the CC-BY Creative Commons Attribution 4.0 International License from reference [118].
tumor cells was also extensively studied by Hume et al. [126], who engineered adipose tissue in a collagen structure to recapitulate breast tissue environment. In this model, CUBIC was applied to first assure uncompromised structure of the generated tissue (using second harmonic generation imaging to detect collagen fibers), and second, it allowed the migratory behavior of tdTomato MDA-MB-231 breast cancer cell line seeded in such scaffolds to be described. This approach was further utilized to demonstrate effect of several chemotherapeutics on migratory tumor cell behavior (represented by e.g. migration distance or number of migratory cells change upon treatment [127]).

Another possible application of TOC to cancer research is in deciphering tumor microenvironment (TME [128]). For instance, Cuccarese et al. [129] reported a significant heterogeneity in the density and infiltration pattern of tumor-associated macrophages in pulmonary tumor model, while Messal et al. [130] precisely analyzed morphological changes during the initial steps of the development of pancreatic cancer. 3D observations revealed two types of neoplastic growth of pancreatic ducts – exophytic and endophytic – in the case of small and large ducts, respectively, showing that epithelial tumorigenesis might be determined by the tension imbalance. To perform successful, multiplex immunostaining of TME, Lee et al. [131] proposed a 3T approach (transparent tumor tomography) that relies on TOC of 400-µm-thick sections with 80% D-fructose solution. This protocol allowed them to present spatial distribution of several markers important in immune TME, such as HER2, Ki-67, CD45, CD31, PD-L1 [131], and spatial pharmacokinetics and distribution of one of the checkpoint inhibitors, anti-PD-L1 antibody, along with its ligand in murine lung and mammary carcinomas [132]. The same method was recently used to examine immune infiltrates of human head and neck tumor biopsies [133]. The TME of breast cancer models was also reinvestigated in pilot studies by several research groups that used TOC of every chemical group, i.e. CUBIC and SeeDB [134], FunGI [60, 135], CLARITY [136], and uDISCO [137]. Besides fluorophore-conjugated trastuzumab and anti-PD-L1 antibodies, other therapeutic agents might be visualized in TME to understand their pharmacokinetics and local distribution and identify relevant biological barriers. In a series of studies, the group led by Chan tested compatibility and optimized TOC (mainly CLARITY approach) to visualize 3D distribution of nanoparticles, a promising group of carriers for antitumor therapeutic agents [138–140]. Based on their initial observations, although CLARITY could efficiently clear several organs of interest, the electrophoresis-driven approach led to substantial, ~30% loss of nanoparticles [138]. Thus, the group optimized the technique in terms of temperature and duration of clearing and resignation from active lipid removal [139] which, coupled with visualization of gold nanoparticles with light scattering [141], allowed them to perform unbiased, artificial intelligence–based profound analysis of interactions between nanoparticles and micrometastases [142] in isolated organs. Micrometastases were already studied in the context of whole mouse body at single-cell resolution with two chemically distinct TOC approaches. First, Kubota et al. [143] described two new CUBIC solutions (CUBIC-L and -R for delipidation and RI matching, respectively) and reported that RI = 1.52 of final RI-matching solution guarantees optimal tissue transmittance. Using this approach, whole-body distribution of several, widely used cancer models were screened down to a single cell level, and quantitative evaluation of few therapeutic agents (i.e. doxorubicin, fluorouracil, cyclophosphamide) on the number and volume of tumor foci was performed. Importantly, the organs cleared with these CUBIC solutions remain compatible with further classical histological analysis, and thus it readily bridges the gap between in vivo live inspection of tumor bioluminescence and 2D histology [143]. Recently, Pan et al. [42] exploited the same idea with vDISCO technology, in which fluorescence of the tumor cells was greatly enhanced using Atto-conjugated antibodies. Such a bright signal allowed the performance of screening of metastases and visualization of tumor-targeting drugs in an automatic, AI-based way with the developed algorithms. Interestingly, the group presented that antibody-based drugs may miss as many as 23% of micrometastatic clusters of tumor cells. It should be mentioned, however, that the majority of the approved anticanter nanoparticle-based therapeutic and experimental formulations are lipid-based [144] (e.g. liposomes, solid lipid nanoparticles, and nanostructured lipid carriers) which are effectively removed with the currently most often used TOC techniques – CUBIC, CLARITY, and DISCO, in particular. To overcome this limitation, Syed et al. [140] developed a tag that, when conjugated with therapeutic liposomes, remains crosslinked to the tissue, even after the liposome undergoes efficient removal. A combination of such approach with the discussed CUBIC or DISCO whole-body clearing might even further extend the applicability of these techniques for preclinical screening of the efficacy of antitumor targeting by emerging compounds.

### Summary

Although valuable for examining literally every biological specimen [4], TOC opens critically important new avenues for immuno-oncology at both basic science and clinical levels. Along with the development of advanced quantitative tools, such as CytoMAP [118], it will be shortly possible to assess the crosstalk between immune and cancer cells during every step of tumorigenesis, extract distinct microenvironmental niches, create their mathematical models, and finally reveal their key components to be targeted during cancer therapy. Moreover, as presented, it could be valuable to evaluate tissue sections of cancer patients in 3D and, possibly, create a new generation of more reliable cancer grading systems. This, however, will require much effort to firstly describe new TOC methods that (1) are rapid and further compatible with standard histopathological pipelines and (2) guarantee comparable results, independently of staff experience; and secondly, to build standardized, easy-to-operate light-sheet fluorescence microscopes.

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