Comparison between optical tissue clearing methods
for detecting administered mesenchymal stromal
cells in mouse lungs

OTC to detect MSCs in mouse lungs

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Summary statement

We present a tissue clearing approach to visualize exogenous MSCs in the mouse lung and study
their effects in the host.
Abstract

Optical tissue clearing of lung tissue enables the intact lung to be imaged using fluorescence microscopy. Several clearing protocols have been developed in recent years, including the Clear, Unobstructed Brain/Body Imaging Cocktails and Computational analysis (CUBIC), stabilised 3D imaging of solvent-cleared organs (s-DISCO) and Ethyl cinnamate (ECi) methods. Here we compared these protocols with the aim of determining the biodistribution of mesenchymal stroma cells (MSCs) and understanding how they interact with host cells in the mouse lung. First, we evaluated how each method affected the size, morphology, and transparency of the lungs. Then, we compared the preservation of the fluorescence of the protein tdTomato expressed by the MSCs, and of the organic dye Evans Blue which labels the vasculature. In addition, we tested the compatibility of the methods with immunofluorescence staining. We found that CUBIC clearing is the only method that enables direct imaging of fluorescently labelled MSCs in the lungs thereby allowing the study of the MSC interaction with endothelial and immune cells when combined with immunofluorescence staining. Overall, 3D imaging of CUBIC cleared lungs confirmed that injected MSCs are initially retained in the pulmonary microvasculature, and that most cells are eliminated from the lungs within the first 24 h.
Introduction

Cell-based therapies include the administration of exogenous cells to trigger a regenerative response. Several sources of therapeutic cells have been investigated (1). The majority of clinical trials have explored the potential of mesenchymal stromal cells (MSCs). Although it has been reported that MSCs are multipotent, their therapeutic effects in vivo are mostly mediated by secreted factors that promote the repair of injured host tissues and modulate the host’s immune system (2). Increasing evidence suggests that the intravenous administration of MSCs and various other cell types is followed by the entrapment of most of the administered cells in the lung capillaries (3–5). Little is known about the role of therapeutic cells in this organ as cell persistence is low, with most cells dying within the first 24h post systemic administration (6).

One of the most common safety issues upon intravascular infusion of MSC therapies are thromboembolic complications (7–9). Thus, it is important to analyse the distribution of the MSCs within the vasculature to determine their potential to occlude the pulmonary vessels (10). Moreover, MSCs can exert their therapeutic effects via immune effector cell mediation triggered by MSC death in the lungs (11), but the interactions of the MSCs with different immune cell populations in the lung remains to be established. Cell tracking by following the biodistribution of labelled therapeutic cells within tissues might offer insights into these questions.

Cell tracking in tissues at single-cell resolution has been traditionally done using thin section histological analyses. Although useful, the field of view is limited and might not be an accurate representation of the whole organ (12). Investigating the biodistribution of cells in thick tissue sections offers an advantage to thin-section analyses (13,14), but biological tissues are dense and inherently scatter light, preventing the visualization of deeper structures (15). Optical tissue clearing is a technique that minimizes the heterogeneities within tissues by removing lipids and matching the refractive index (RI) between the sample and the imaging medium (16). As a result, opaque samples become more transparent and the decrease in light scattering allows deep-tissue imaging (17).

Optical tissue clearing, in combination with molecular labelling and optical sectioning microscopy, has become an important tool for 3D imaging in several biological
applications including investigating the biodistribution of cells in whole organs (18,19).

Despite the many advantages of optical tissue clearing, practical limitations to the applicability of clearing protocols for imaging remain. Several protocols have been developed in recent years (20) and selecting between them requires careful consideration of a range of parameters to achieve the optimal trade-off for specific applications. Sample size and tissue composition impact the clearing speed and limit the microscopes that can be used when imaging large samples (21). Fluorophore preservation poses another challenge as certain clearing protocols are incompatible with fluorescent probes (22). In particular, preservation of protein-based fluorescence and lipid staining remain open challenges in the field (16). The compatibility of immunostaining with the chemicals used for clearing as well as antibody penetration in large samples requires testing and optimization (21). Moreover, certain parameters differ between tissue and sample types, and no single clearing approach fits all, necessitating the use of application/tissue specific protocols.

Several studies have focused on clearing lung tissue to investigate biological processes (23–25). An example is the study of blood vessel formation, achieved by labelling the intact vasculature with the organic dye Evans Blue (26). Nevertheless, no study to date has performed optical tissue clearing to investigate the biodistribution of administered MSCs within the lungs. Here, we compared three different tissue clearing protocols: CUBIC, s-DISCO and ECi. CUBIC was chosen based on its ability to preserve fluorescent proteins (27); ECi due to its cost-effectiveness, safety and ease of access to the reagents required (28); and s-DISCO due to its reported compatibility with fluorescent proteins despite being a solvent-based method (26).

The comparison of these methods was performed with the aim of imaging thick lung slices in 3D to track MSCs labelled with the genetic reporter tdTomato and study their biodistribution within the host’s lung. Moreover, as a proof of principle, we explored the possibility of using optical tissue clearing to investigate the interactions of the administered MSCs with the host’s endothelial cells and immune microenvironment.
Methods

Cell culture and stable cell line generation

Human umbilical cord-derived mesenchymal stromal cells (hUC-MSCs) were obtained from the National Health Service Blood and Transplant (NHSBT, UK) at passage 3. The hUC-MSCs were transduced in the presence of 6 µg/ml DEAE-Dextran with a lentiviral vector pCDH-EF1-Luc2-P2A-tdTomato, encoding luc2 firefly luciferase (FLuc) reporter under the constitutive elongation factor 1-α (EF1α) promoter and upstream of a P2A linker followed by the tdTomato fluorescent protein (gift from Kazuhiro Oka; Addgene plasmid # 72486; http://n2t.net/addgene:72486; RRID:Addgene_72486). To obtain a >98% transduced population, the cells were sorted based on tdTomato fluorescence (BD FACS Aria). Cells were cultured in α-MEM supplemented with 10% FBS at 37°C and in 5% CO₂, and passaged at 80% confluence.

Animal experiments

Eight to ten-week-old female albino mice (C57BL/6) (B6N-Tyr^C-Brd^BrdCrCr1 (n=15), originally purchased from the Jackson Lab) were used for all animal experiments. Mice were housed in individually ventilated cages (IVCs) under a 12 h light/dark cycle and provided with standard food and water ad libitum. All animal procedures were performed under a licence granted by the Home Office under the Animals (Scientific Procedures) Act 1986 and were approved by the University of Liverpool Animal Welfare and Ethics Review Board. Mice received 2.5 x 10⁵ FLuc-tdTomato-hUC-MSCs (hUC-MSCs hereinafter) suspended in 100 μL of PBS by intravenous (IV) administration via the tail vein under inhaled anaesthesia with isoflurane.

Bioluminescence imaging

In vitro bioluminescence was performed by seeding a range of cell densities (from 625 to 2 x 10⁴ cells/well) into an optical bottom 96-well plate with black walls (#165,305, ThermoFisher). The cells were allowed to attach for 3 h prior to the addition of 5.12 mM D-Luciferin. Imaging was performed immediately after substrate addition without an emission filter, a 13.3 cm field of view (FOV), f-stop of 1 and a binning of 8. For in vivo bioluminescence imaging, mice received a subcutaneous (SC) injection of D-Luciferin (10 μL/g [body weight] of a 47 mM stock solution) after
cell injection. After 20 min, the animals were imaged with an IVIS Spectrum instrument (Perkin Elmer). Data are displayed in radiance (photons/second/centimeter²/steradian), where the signal intensity scale is normalised to the acquisition conditions. Acquisition was performed without an emission filter, a 22.8 cm FOV, f-stop of 1 and a binning of 8.

**Tissue preparation**

Immediately after bioluminescence imaging (BLI), the animals received an IV injection of Evans Blue (Sigma; 3 µl/g) for vascular labelling. The dye was allowed to circulate for 5 min before proceeding with a retrograde perfusion fixation protocol (25). The animals received an intraperitoneal overdose of pentobarbital (Pentoject, 100 µl) followed by cannulation of the abdominal aorta, opening of the vena cava and flushing PBS with a manual pump at a constant pressure of 200 mbar (supplementary figure 1) for 6 min, to remove all blood cells, followed by 6 min perfusion with 4% paraformaldehyde (PFA) to fix the whole animal. The total volume of each solution used per animal was 40 ml. The trachea was tied tightly with a surgical suture before opening the thoracic cavity for lung dissection. Finally, the lungs were post-fixed in 4% PFA overnight at 4°C.

**Optical tissue clearing**

Solvent-based tissue clearing: s-DISCO and ECi

The general procedure consists of dehydrating fixed samples by sequentially adding pH9-adjusted solvents chilled to 4°C. After dehydration, the respective RI matching solution was added until the samples reached optimal transparency for 3D imaging (28,30). Solvents used and incubation duration are detailed in table 1.
Table 1. Solvent based clearing protocols. The RI matching solvents used were Ethyl cinnamate (ECi), Dichloromethane (DCM), and Dibenzy ether (Dibenzy ether [DBE] - Sigma-Aldrich).

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Aqueous-based tissue clearing: *Clear, Unobstructed Brain/Body Imaging Cocktails and Computational Analysis* (CUBIC)

The CUBIC-cancer protocol was followed (31). Briefly, CUBIC-L2 solution (L2), for delipidation and decolourisation, was prepared as a mixture of 10 w%/10 w% Triton X-100 (Sigma-Aldrich)/N-buthyldiethanolamine (B0725 Tokyo Chemical Industry). CUBIC-R2 solution (R2), for RI matching (RI=1.52), was prepared as a mixture of 30% (w/v) nicotinamide (Sigma) and 45% (w/v) antipyrine (Sigma).

For whole-organ clearing, 4% PFA fixed lungs were washed with PBS for 2 h, three times each, followed by immersion in CUBIC-L1 solution (L1) (50% (v/v) mixture of water and CUBIC-L2) for 6 h at 37°C. Then, the organs were immersed in L2 solution at 37°C for 48 h. L2 solution was refreshed after 24 h during this process. After decolourisation and lipid clearing, the organs were washed with PBS at room temperature for 2 h, followed by immersion in CUBIC-R1 solution (R1) (50% (v/v) mixture of water and CUBIC-R2) for 6 h at room temperature. Finally, organs were immersed and stored in R2 solution at room temperature overnight.
**Size change and transparency measurements**

Fixed adult mouse lungs were cleared and imaged before and after clearing. The size and transparency of the samples were outlined and calculated using ImageJ (by drawing the region of interest and measuring mean pixel intensity) (NIH, USA) (32). The median grey value of the cleared organ image was used to measure transparency by normalizing the obtained value to the background of the same image (33).

\[
\text{Transparency} = \frac{\text{sample median grey value}}{\text{average background median grey value}} \times 100
\]

**Immunofluorescence**

The lungs were cryoprotected in 15% sucrose followed by 30% sucrose over a period of 48 h before embedding in optimal cutting temperature (OCT) medium. The samples were cut into 500 µm sections on a cryostat (Thermo Scientific, Microm HM505E) at -20°C and stored at -80°C.

For CUBIC staining, the tissue sections were delipidated by immersion in 50% CUBIC-L for 30 min at 37°C followed by overnight incubation in CUBIC-L at 37°C with shaking. All sections were washed 3x with PBS for 5 min. Tissues were incubated with zeron Alexa Fluor® 647 (Invitrogen, Z25008) labelled human mitochondria primary antibody (Merck, MAB1273), 1:500 for 48 h at 4°C and washed with PBS overnight at 4°C. For CD31 (R&D systems, AF3628) staining, the lung sections were blocked overnight with PBS-TxDBN buffer (1x PBS, 2% TritonX-100, ddH₂O, 2% BSA, 20% DMSO) at 37°C and incubated with CD31 1:100 antibody for 72 h at 37°C. Upon an overnight washing step, secondary antibody (Alexa Fluor® 647) incubation was done at 37°C for 24 h. After the final overnight washing step, solvent-based or CUBIC clearing was performed as indicated in the clearing section.

**Imaging**

Cells in culture were imaged by light microscopy with a Leica DM IL microscope coupled to a DFC420C camera. Confocal images were acquired on a Leica DMi8 with Andor Dragonfly spinning disk, coupled to an EMCCD camera using a 10x/0.45 air objective. Z-stacks were captured using the 488, 561 and 637 nm laser lines. The
emission filters used were 525/50, 600/50 and 700/75. Maximum intensity projections, three-dimensional reconstructions and image analysis were done using the IMARIS (Bitplane) software packages, processed with ImageJ 3D viewer (32). 3D surfaces were obtained via un-stacking the .ims image in Image J and reconstructed via variable threshold intensity (3D slicer). 3D elaboration was performed using NVIDIA ® Quadro 6000 GPUs, and exported as .stl.

**Statistics**

The GraphPad Prism software was used to conduct the statistical analysis. The mean and standard deviation are used to represent all values in graphs. The number of replicates included in the analyses, as well as the type of statistical test used, are given in the figure legends.

**Data availability**

The data that support the findings of this study are available to download from Zenodo at [http://doi.org/10.5281/zenodo.6638775](http://doi.org/10.5281/zenodo.6638775)
Results

**Bioluminescence imaging reveals hUC-MSC entrapment in the lungs**

To monitor the fate of MSCs administered in mice, the cells were stably labelled with fluorescent and luminescent reporters. The tdTomato reporter was used as it is the brightest red shifted fluorescent protein available, helping overcome the high autofluorescence of tissues (figure 1a, left) (34). In vitro, the level of emitted bioluminescence is dependent on the number of hUC-MSCs present (figure 1a, right). Immediately after IV administration of hUC-MSCs their localisation was monitored in vivo using bioluminescence imaging, allowing us to observe that the hUC-MSCs were localised to the lungs (figure 1b).

**Figure 1. In vitro and in vivo imaging of hUC-MSCs.** a) Transmitted (top) and epifluorescence (bottom) images of transfected hUC-MSCs in culture. Scale bar = 100 µm (left). Representative image of hUC-MSCs seeded at decreasing concentrations (from $2 \times 10^4$ to 625 cells/well) and treated with 5.12 mM D-Luciferin (right). b) In vivo biodistribution of $2.5 \times 10^5$ hUC-MSCs 20 min after intravenous administration.
Lung clearing comparison after different optical tissue clearing methods

Whilst bioluminescence is useful for monitoring the whole body biodistribution of hUC-MSCs in vivo, the low spatial resolution precludes a detailed analysis of the cell distribution within the lung. To better understand why the cells were retained in the lungs, and to analzye their impact on the host at the molecular, cellular and tissue level, we sought to monitor cell biodistribution in the lung tissues ex vivo at single cell resolution. To achieve this, we compared three different optical tissue clearing methods that would enable the cells to be visualised in thick lung sections; CUBIC, s-DISCO and ECi. S-DISCO and ECi are solvent-based methods that cleared whole mouse lungs in a matter of hours, while CUBIC uses water-based reagents and required several days to entirely clear tissues with an average lipid-clearing of 3 days (figure 2a).

To reach transparency, the samples were immersed in RI matching solutions and representative images of the cleared lungs are shown in figure 2b. We quantified the resulting levels of transparency from digital images taken of the tissues before and after clearing. The CUBIC protocol resulted in the highest transparency of the lungs, reaching close to 90%, while s-DISCO and ECi demonstrated transparency of approximately 30% (figure 2c). Additionally, we calculated the size change and found that there was a significant increase in size after CUBIC clearing. The opposite was observed after s-DISCO and ECi clearing, where the samples shrunk (figure 2d). We observed the presence of adequate air spaces, bronchioles, alveolar sacs, and blood vessels in images acquired by recording tissue autofluorescence. Overall, this indicates that tissue morphology remains unaffected by all the clearing protocols (figure 2e).
Figure 2. Comparison of the effect of different clearing methods on lung tissue. a) Timeline required to clear whole lungs using each protocol. L1 = Delipidation and decolourisation cocktail 50% (v/v). L2 = Delipidation and decolourisation cocktail 100%. R1 = RI matching cocktail 50% (v/v). R2 = RI matching cocktail 100%. Percentages in s-DISCO and ECI represent the solvent concentration. b) Representative images of lungs before (top row) and after (bottom row) optical tissue clearing. Each square represents 2 mm. c) Quantification of transparency of lung samples after optical tissue clearing [33]. One-way ANOVA with Tukey’s multiple comparisons test \( p < 0.05 n = 3 \). d) Size change of lungs before and after each tissue clearing method was evaluated using multiple paired t-tests \( p < 0.05 n = 3 \). e) 3D variable threshold intensity surface reconstruction of lung sections cleared by the different protocols. CUBIC, s-DISCO and ECI cleared lungs show a normal lung morphology after optical tissue clearing. Large blood vessels are indicated by asterisks (*), airways are indicated by arrowheads. Processing artefacts, due to tissue dehydration, are observed as cracks in the ECI cleared sample. 3D MIPs before reconstruction can be viewed in supplementary figure 2.
Effect of different optical tissue clearing methods on the preservation of fluorescence in the lungs

The ability to detect fluorescent dyes and fluorescent proteins is essential to study the environment and fate of the injected cells, yet fluorescence quenching is one of the key limitations of many organic solvent-based optical tissue clearing methods. Therefore, we compared the effect of CUBIC, s-DISCO and ECi on the tdTomato fluorescent label intensity of the hUC-MSCs. By acquiring fluorescent images of cleared 500 µm lung sections immediately after clearing and 3 days after the samples had been stored in RI matching medium, we found that CUBIC preserved tdTomato fluorescence upon all clearing steps and that storage did not affect the fluorescence of tdTomato. On the other hand, the solvent-based methods increased background autofluorescence, and also appeared to quench the tdTomato fluorescence (figure 3A). Interestingly, clearing thinner lung sections (100 µm) using a reduced dehydration time (supplementary table 1) allowed the detection of tdTomato immediately after both, s-DISCO and ECi, indicating that dehydration time is a key parameter in preserving the fluorescence of proteins. Moreover, storage of the thin sections in ECi for 3 days did not result in tdTomato quenching, but storage in dibenzylether (DBE) in the s-DISCO protocol did (supplementary figure 3).
Systemic cell administration results in a large fraction of the cells becoming entrapped in the lung’s vasculature (10). Since Evans Blue allows the labelling of the vasculature to analyse IV administered hUC-MSCs in the context of the pulmonary 3D vascular network, we determined whether Evans Blue labelling was affected by the clearing protocols. Our analysis showed that Evans Blue is incompatible with CUBIC since all fluorescent signals were lost (figure 3b). By contrast, Evans Blue fluorescence was not only preserved by s-DISCO and ECi, but the fluorescence intensity of the dye had increased after clearing with either solvent-based method. We speculate that the shrinkage of the samples leads to a higher density of fluorescent molecules and subsequent increase in signal intensity, since the samples were imaged under the same conditions.
**Compatibility of antibody labelling with different clearing methods**

Although the tdTomato fluorescence of hUC-MSCs was not detectable in s-DISCO- and ECi-treated thick lung sections, the biodistribution of administered cells can be investigated using cell-specific antibodies. To test the compatibility of each optical clearing method with antibody staining, we aimed to detect the hUC-MSCs with a specific antibody within the lungs. In this instance, we utilised the human origin of the hUC-MSCs and applied an antibody specific for human mitochondria to distinguish the hUC-MSCs from the mouse tissue. Alternatively, antibodies against the tdTomato or FLuc reporters could be applied.

Our image analysis showed that the human mitochondria antibody colocalizes with the tdTomato signal in CUBIC cleared samples confirming the specificity of this antibody to the human hUC-MSCs (figure 4a). Moreover, we observed that the antibody signal was detected throughout the 500 µm thick sample. Similarly, in the s-DISCO and ECi cleared samples, the antibody permeated the entire tissue (figure 4b, c).
Figure 4. Preservation of immunofluorescence after optical tissue clearing. The human mitochondria antibody (white) was used to stain 500 µm lung sections followed by clearing with either CUBIC, s-DISCO or ECi. The antibody penetrated the entire depth in all samples. a) 3D z-stack of a CUBIC cleared lung section (left). Maximum intensity projection and single slices at different sample depths (right). b) 3D z-stack of an s-DISCO cleared lung section (left). MIP and single slices at different sample depths (right). c) 3D z-stack of an ECi cleared lung section (left). MIP and single slices at
different sample depths (right). hUC-MSCs are indicated by arrowheads. Scale bar = 200 µm (left), 150 µm (right).

In vivo and ex vivo tracking of administered hUC-MSCs by BLI and optical tissue clearing

To evaluate the biodistribution and fate of hUC-MSCs in vivo, the animals were imaged using BLI immediately after IV administration of the cells, and 24 h post injection. A strong signal was detected in the lungs on the administration day but was significantly reduced on day 1 (figure 5a, b). We dissected whole lung lobes on the day of cell injection and at day 1, and performed CUBIC clearing, as this was the method that allowed the direct detection of the hUC-MSCs. Subsequent confocal imaging of whole lung lobes revealed that the cells distributed throughout the tissue and did not home preferentially to any site. Ex vivo imaging of CUBIC-cleared lungs collected on day 1 after cell administration confirmed that most of the cells had been cleared from the lungs as shown by the reduced size of the cell clumps as well as the overall cell distribution (figure 5c).

Given the compatibility of the CUBIC protocol with immunofluorescence, as a proof of concept, we explored the possibility of using this clearing method to study the interaction of the hUC-MSCs with cells of the mouse lung. The vasculature was labelled with the CD31 endothelial marker, which showed that the hUC-MSCs appear to be retained in the pulmonary microvasculature as no cells were detected in the interstitium (figure 5d).

Alternatively, the vasculature can be labelled by injecting Evans Blue IV. In the experiment described previously (figure 4), the Alexa Fluor® 647 secondary antibody was used, but its spectrum overlaps with Evans Blue. Due to an increase in tissue autofluorescence across all wavelengths, following ECI, we were unable to label the hUC-MSCs utilising the green, red, or near infrared channels. Thus, it was not possible to immunolabel the hUC-MSCs in lungs stained with Evans Blue and cleared by ECI. Nevertheless, thin section analysis of uncleared lungs stained with Evans Blue, by injecting dye IV after cell injection, revealed that hUC-MSCs remained in close contact with the pulmonary vasculature when the lungs were harvested immediately after cell administration (Supplementary figure 4a). In addition, hUC-MSCs blocked the free flow of Evans Blue dye as evidenced by the
accumulation of dye around areas where the hUC-MSCs are present. Moreover, the lack of Evans Blue vascular staining in lung regions surrounded by hUC-MSCs suggested that the cells had formed emboli (35) (Supplementary figure 4b).

Finally, to demonstrate the usefulness of the CUBIC clearing protocol to study immune responses in the lung after hUC-MSC administration, neutrophils were stained with the myeloperoxidase (MPO) marker. A rapid neutrophil infiltration was observed 2 h after IV hUC-MSC injection with decreasing neutrophil levels after 24 h (figure 5e).
Figure 5. In vivo and ex vivo imaging to detect hUC-MSC distribution for up to 24 h post-administration. 

a) $2.53 \times 10^5$ hUC-MSC were injected via the tail vein and the mice were imaged on the administration day (Day 0) and 24 h post cell administration (Day 1). Representative images of the mice as acquired 20 min post subcutaneous administration of D-Luciferin. 

b) Flux (light output) as
a function of time. Data are displayed as mean ± SD from n = 4. Statistical analysis was performed using a paired student T-test. **p < 0.05. c) hUC-MSC biodistribution in whole mouse lung lobes after CUBIC clearing on the day of cell administration and 24 h post injection. Scale bar = 800 µm. d) CD31 stained 500 µm lung section on the day of administration and 24 h post cell injection. Large vessels are indicated by arrowheads. e) Neutrophil recruitment to the lungs 2 and 24 h after hUC-MSCs administration. MPO = Myeloperoxidase. Scale bar = 100 µm.

In summary, of the three methods compared, the CUBIC protocol proved to be suitable for efficiently clearing the lung specimens without altering the tissue morphology. CUBIC was the only method that preserved tdTomato fluorescence in thick lung sections, allowing for direct visualisation of hUC-MSCs by confocal microscopy. However, CUBIC failed to preserve Evans Blue labelling of the vasculature but showed good antibody compatibility. In contrast, s-DISCO and ECi allowed rapid optical clearing of whole lungs but permanently quenched the fluorescence of tdTomato, while preserving the endothelial Evans Blue signal. Despite the fact that no clearing method was perfect for all subsequent imaging applications, by combining the CUBIC clearing method with various staining approaches, we were able to demonstrate that hUC-MSCs were trapped in the lung microvasculature just after injection, potentially blocking blood flow, and that hUC-MSC intravenous administration triggers neutrophil infiltration.
Discussion

In this work, the CUBIC, s-DISCO and ECi optical tissue clearing protocols were compared with the aim of establishing a suitable approach to study the biodistribution of hUC-MSCs in mouse lungs following systemic cell delivery.

Optical tissue clearing matches the RI of heterogeneous samples and reduces light scattering, enabling the investigation of biological processes in a whole organ context. Broadly, clearing methods can be classified into water-based (hydrophilic) and solvent-based (hydrophobic) methods depending on the chemistry used (20). Selecting a clearing protocol depends on parameters such as the size of the sample, tissue composition and the intended goal of the experiment.

First, it is relevant to characterize the effect of the clearing method on the tissue of interest. When applied to lung tissue, CUBIC is the superior method regarding tissue transparency albeit taking 5 days to complete. In contrast, both solvent-based methods cleared the samples within hours but resulted in a transparency below 50%. Changes in sample size occurred as expected: hydrophilic methods led to sample expansion, which might be an advantage when interested in increasing imaging resolution; solvent-based protocols resulted in sample shrinkage, which can be advantageous when imaging large samples (19).

Another relevant consideration when selecting a clearing protocol is whether there is a need to preserve fluorescent proteins. In this study, the red fluorescent protein tdTomato was used as a molecular label for the hUC-MSCs. tdTomato was immediately quenched after s-DISCO and ECi clearing of thick lung sections, rendering these protocols unsuitable for direct visualization of fluorescently labelled cells, while CUBIC preserved the fluorescence of tdTomato.

Fluorescent proteins are stabilized by water molecules and thus, dehydration results in their denaturation and subsequent loss of fluorescence (36). To overcome this, the use of milder dehydrating chemicals such as tetrahydrofuran (THF) in the 3DISCO protocol (37), tert-butanol and diphenyl ether in the uDISCO protocol (38), and 1-propanol in the second generation ECi protocol have been used successfully allowing GFP preservation for days to months (28). Given these reports, we used 1-propanol as the dehydration agent for both s-DISCO and ECi protocols in the hope of preserving tdTomato. Nevertheless, we were not able to detect tdTomato.
fluorescence. Interestingly, Glaser and colleagues acquired a 3D image of a whole mouse lung cleared with ECi via autofluorescence at 561 nm, which is consistent with our observation that tissue autofluorescence at this wavelength is increased after immersion in ECi (39). Moreover, the compatibility of a variety of fluorescent probes with ECi was tested and tdTomato fluorescence was reported to be poor after clearing (28).

In this study, the s-DISCO protocol was chosen given that the protocol suggests that adding the antioxidant propyl gallate to DBE prevents the formation of peroxides and aldehydes, which are partly responsible for fluorescence decline, and makes it possible to preserve tdTomato for up to a year. We did not observe fluorescence preservation after s-DISCO clearing. A possible explanation for this difference is that Hahn, et al. purified the dehydration chemical tetrahydrofuran and DBE to eliminate all peroxide and aldehyde contents before adding the propyl gallate to prevent their regeneration (30). This indicates that simply preventing new peroxide formation is not sufficient to preserve fluorescent proteins and suggests that pure chemicals might be necessary throughout the clearing protocol (30). The purification protocol requires specific expertise and great care given that the chemicals used are explosive.

Interestingly, we observed that the fluorescence of tdTomato was preserved immediately after the completion of the s-DISCO and ECi protocols by decreasing the dehydration time when clearing 100 µm thin lung sections. This finding might explain the variability in the results between users of the same protocols for different applications and samples (40). Moreover, it indicates that optimizing the duration of the dehydration steps might be necessary when implementing a solvent-based optical tissue clearing method where fluorescent proteins are involved. Decreasing the dehydration time might result in protein-based fluorescence preservation but it would come at a cost regarding sample transparency. Moreover, dehydration is not the only factor that affects tdTomato as evidenced by the preservation of fluorescence upon storage in ECi, but not in DBE, indicating that the RI matching solvent also plays a critical role in fluorescence quenching.

Temperature and pH have also been proven to play an important part in fluorescence retention, with alkaline pH and 4°C being the optimal parameters that
allow fluorescent protein preservation. The studies that showed this were done specifically to preserve GFP fluorescence (37,41,42). Although we followed these recommendations, we failed to visualize tdTomato fluorescence.

Preservation of other fluorescent compounds, such as synthetic organic dyes, is another consideration when selecting a tissue clearing method for a particular application. To study hUC-MSC distribution with spatial landmarks in the 3D lung context, the vasculature was labelled with Evans Blue. CUBIC washed away the dye, while s-DISCO and ECi preserved Evans Blue fluorescence.

In summary, CUBIC is the only protocol that preserved tdTomato but it resulted in Evans Blue washing out of the sample. The opposite occurred when using s-DISCO or ECi, as tdTomato was quenched, but Evans Blue was preserved. These results reflect the challenges of optimizing a clearing method for a specific application. Nevertheless, using immunofluorescence for the endothelial marker CD31 to label the vasculature in CUBIC-cleared lung sections indicated that the hUC-MSCs localise within the micro vessels and do not seem to migrate to the parenchyma after 24 h.

Finally, we briefly explored the possibility of using CUBIC to study the immune response in the lung to the administration of hUC-MSCs. A rapid infiltration of neutrophils was observed 2 h post cell injection with the number of these cells decreasing after 24 h. This proof of principle paves the way for studying other immune cell populations in thick lung sections in the context of cell therapies.

**Conclusions**

We compared three optical tissue clearing methods previously described in the literature to track the lung biodistribution of hUC-MSCs labelled with the fluorescent protein tdTomato. Direct detection of the tdTomato cells was only possible using the CUBIC clearing protocol, which although time consuming, results in highly transparent lungs and showed good antibody compatibility and penetration. Moreover, using immunofluorescent staining allows the study of the interaction of the hUC-MSCs with cells in the host's lung. Using 3D imaging of CUBIC cleared lungs we showed that hUC-MSCs were trapped in the pulmonary vasculature and are mostly cleared within the first 24 h after IV injection.
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Conflicts of interest

The authors declared that they have no conflicts of interest to this work.
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