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SHORT COMMUNICATION

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Development of a protocol for maintaining viability while shipping organoid-derived retinal tissue

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Abstract

Retinal organoid technology enables generation of an inexhaustible supply of three-dimensional retinal tissue from human pluripotent stem cells (hPSCs) for regenerative medicine applications. The high similarity of organoid-derived retinal tissue and transplantable human fetal retina provides an opportunity for evaluating and modeling retinal tissue replacement strategies in relevant animal models in the effort to develop a functional retinal patch to restore vision in patients with profound blindness caused by retinal degeneration. Because of the complexity of this very promising approach requiring specialized stem cell and grafting techniques, the tasks of retinal tissue derivation and transplantation are frequently split between geographically distant teams. Delivery of delicate and perishable neural tissue such as retina to the surgical sites requires a reliable shipping protocol and also controlled temperature conditions with damage-reporting mechanisms in place to prevent transplantation of tissue damaged in transit into expensive animal models. We have developed a robust overnight tissue shipping protocol providing reliable temperature control, live monitoring of the shipment conditions and physical location of the package, and damage reporting at the time of delivery. This allows for shipping of viable (transplantation-competent) hPSC-derived retinal tissue over large distances, thus enabling stem cell and surgical teams from different parts of the country to work together and maximize successful engraftment of organoid-derived retinal tissue. Although this protocol was developed for preclinical in vivo studies in animal models, it is potentially translatable for clinical transplantation in the future and will contribute to developing clinical protocols for restoring vision in patients with retinal degeneration.

KEYWORDS

retinal organoids, shipping, subretinal, surgery, transplantation, vision restoration

1 | INTRODUCTION

Shipping and storage of three-dimensional (3-D) retinal tissue dissected for transplantation into the subretinal space of animal models with retinal degeneration and for clinical trials in patients with retinal degeneration (CT NCT00346060 and NCT00345917) have previously utilized Hibernate E medium. This has allowed for successful engraftment of viable tissue (Radtke et al., 2008; Seiler & Aramant, 2012). Retinal tissue derived from human pluripotent stem cells (hPSCs) is similar to the transplantable human fetal retinal tissue that was used

in these clinical trials. The organoid-derived retinal tissue has the distinct advantage that it can be produced in limitless quantities at different stages of retinal development and is currently viewed as a good candidate for designing a 3-D retinal "patch" to ameliorate vision loss in advanced retinal degeneration conditions such as age-related macular degeneration and retinitis pigmentosa (Lin, Mclelland, Mathur, Aramant, & Seiler, 2018; Singh et al., 2015; Singh et al., 2018) and from retinal trauma. To model these therapies, retinal organoids need to be derived in a stem cell lab and then shipped to the surgical site for implantation into animal models. Compared with the logistics of shipping human fetal retinal tissue (which needs to be shipped at low temperature and stored at 4°C; Seiler et al., 2008), retinal organoids are in vitro generated retinal tissue growing in tissue culture media at 37°C, and they need no dissection before shipment and benefit from being shipped under conditions identical or similar to the tissue culture incubator in the originating stem cell laboratory. Maintenance of viability and sterility of organoids while shipping and detecting any potential trauma to the tissues ensures that they are delivered with maintained viability and ready to implant. This also minimizes variability from experiment to experiment and maximizes chances of optimizing retinal tissue viability for subretinal grafting facilitating the development of preclinical protocols modeling retinal tissue implantation in the ocular space for ameliorating vision loss.

Various federal agencies developed guidelines, rules, and regulations for transportation of biological (especially human) specimens, including Transportation Safety Administration, Federal Aviation Administration, Department of Transportation, Centers for Disease Control, Food and Drug Administration, and a number of others. Because of this, a specialized set of contracted courier companies evolved driven by the market need to rapidly transport biological samples via large distances. We were faced with these logistical challenges while developing a project for large-scale human embryonic stem (hPSC)–derived retinal tissue testing in vivo in the animal models with retinal degeneration. We were initially offered services of World Courier, which is often used for transporting organs for actual transplantation surgeries in patients. However, at $2,000 or more per shipment and the need for optimization of multiple steps (starting from developing understanding between shipping and receiving teams with regard to handling the organoids, working out surgical protocol and development stage of hPSC retinal tissue), this was a very costly option. Therefore, we developed a simple but very efficient and robust retinal organoid shipment protocol and a 37°C portable shipping container (Figure 1), which is recognized and accepted by FedEx. This method saves about 85% per shipment (with an average cost of $300, including the cost of shipping it back by second-day delivery). The protocol minimizes transportation time, maximizes the viability and vitality of hPSC retinal tissue for preclinical experiments, is economical, and has been used dozens of times by our collaborating teams over a 2-year period without failure, including when shipping organoids from California to cold states in winter (e.g., Michigan).

We remove retinal organoids from a stem cell incubator at about 4:00–4:30 p.m. local time, place in a 15-ml tube filled with culture medium (Singh, Occelli, Binette, Petersen-Jones, & Nasonkin, 2019) pre-saturated (for 1–2 hr, in the same incubator) with optimized gas concentrations (CO_2 and oxygen), place the tube in the shipping container (preequilibrated to reach 37°C), which is then placed in a shipping crate recognized by FedEx, with signs "do not X-ray" and "keep in the upright position," and deliver the crate to a nearby FedEx shipping facility about 30–45 min before the designated cutoff pickup time, which may vary between the locations and also depends on the shipping destination (see protocol below). We use three tracking devices: two inside the shipper—each reporting temperature conditions inside the shipper, and one additionally reporting the Global Positioning System (GPS) location and the g-forces inside the shipper; one tracker is placed outside the shipper to monitor temperature conditions experienced by the 37°C shipping container inside the shipping crate. The shipping temperatures drop close to freezing temperatures outside the shipper when shipping in winter (outside tracker), whereas inside the container, the temperature fluctuates between 33°C and 36°C (which we found acceptable and not impacting viability of hPSC retinal tissue). The package is delivered to the designated location by 10:30 a.m. (although 7:30 a.m. delivery time is also an option, if the organoids are to be transplanted on the same day), and following delivery, the organoids are placed in a tissue culture incubator (either on a shaker or into ultra-low adhesion dishes) until their use in surgery (typically in 1–2 days after delivery). Collectively, our approach maximizes the viability of retinal organoids for subsequent surgical procedures, optimizes the environment for the organoids during shipping, and enables us to identify if the package suffers severe g-forces during delivery that could damage the retinal organoids. This reduces the risk of using compromised tissues for implantation, thus optimizing the outcomes, saving valuable animals and resources, and, collectively, streamlining the development of preclinical protocols aimed at restoring vision by retinal tissue replacement.

2 | DESIGN OF PORTABLE SHIPPER

We use a battery-driven portable shipper with 0.028-m^3 (U.S. 4 ft^3) capacity (manufacturer: Uline, Part Number S-20589, Model Number 20589), external dimensions [L × W × H] 70.87 × 52.3 × 38.74 cm (U.S. 27.9 × 20.6 × 15.25 in.), and internal dimensions 66.04 × 48.26 × 35.56 cm (U.S. 26 × 19 × 14 in.; Figure 1b). We also use two types of tracking devices: the multiuse SpotBot Cellular (ShockWatch, Australia; Figure 1c) and multiuse TempTale Ultra (Sensitech, Beverly, MA, USA; Figure 1d), to report the Global Positioning System (GPS) location, g-forces, and temperature remotely (SpotBot) and temperature only (as a backup, TempTale Ultra). The SpotBot tracker allows live monitoring of the container's location (except when it is on the plane) and proactive tracking if it was subjected to excessive g-forces and even the direction of these forces (e.g., vertical vs. horizontal shaking, Figure 1h). Although it is not critical for organoid shipment (because they are shipped as a suspension culture), it may negatively impact the adherent monolayer cultures of retinal progenitors. The monolayers of retinal (and any neural) progenitors differentiated from

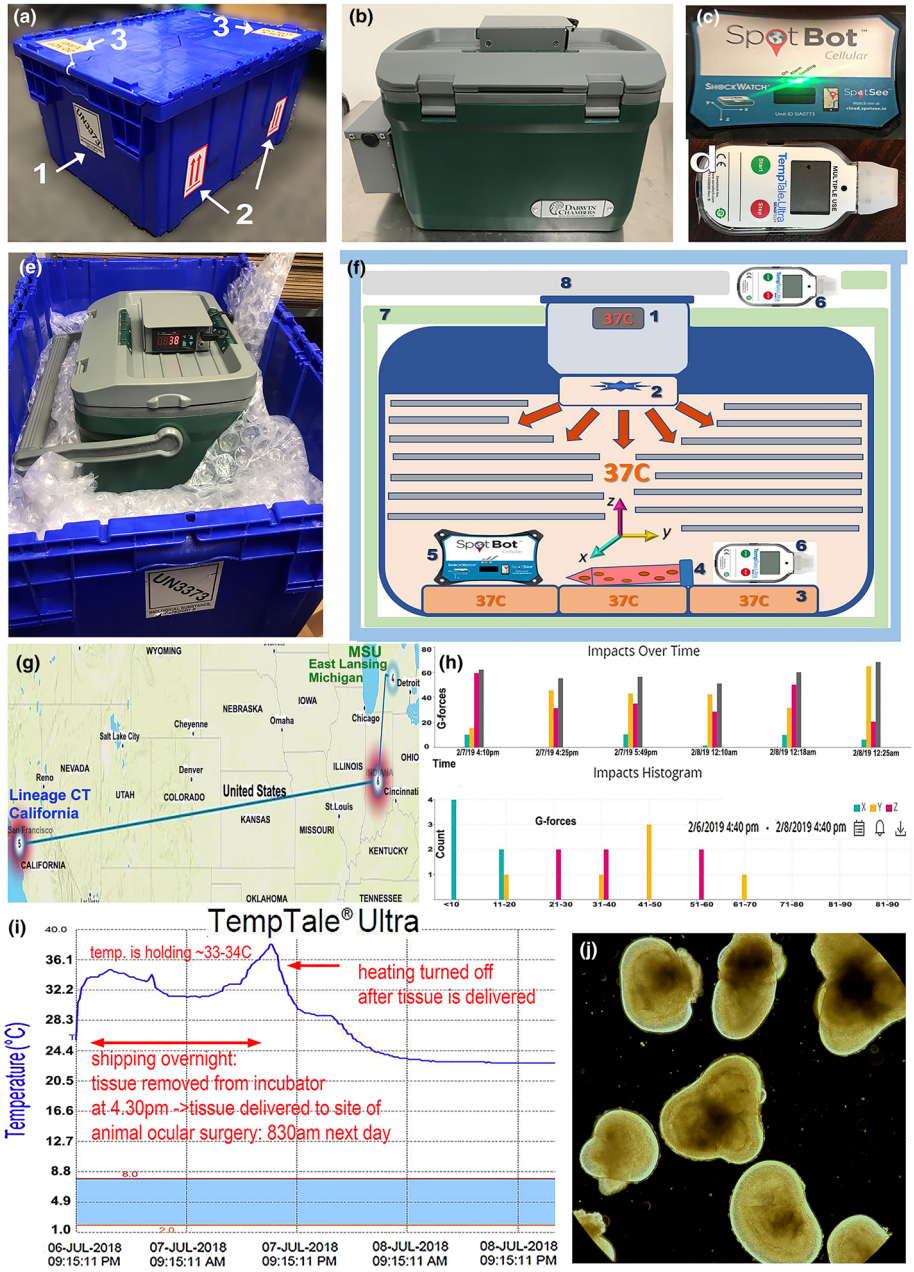

FIGURE 1 37°C Shipping container and trackers. (a) Shipping crate with mandatory sticker "UN3373" (Category B biological substance), "do not X-ray," and "up" arrows (to emphasize package orientation). (b) Portable 37°C incubator from Uline, Part Number S-20589, Model Number 20589. (c) SpotBot live temperature, GPS, and g-force tracker, which allows live monitoring of the package location, temperature conditions, and g-forces acting in the *x*-*y*-*z* orientations. (d) Multiuse TempTale Ultra temperature tracker, which enables analysis of the detailed temperature profile changes during each shipment but only after data are downloaded (needs company's software). (e) Portable 37°C incubator from Uline in a shipping crate. (f) Diagram of a portable 37°C incubator from Uline prepared for shipment (f-1, motor housing; f-2, temperature reader, sensitive to mechanical damage; f-3, four to six Cameron's Hot/Cold Gel Pak™, which absorb 37°C temperature during incubator-prewarming step [ahead of shipment] and then provide temperature backup if the battery runs out of power on an especially cold day; f-4, 15-ml tissue culture tube with retinal organoids, loaded with CO_2- and oxygen-presaturated media [the same media and gas conditions used for organoid culture] and placed in horizontal position, maybe further secured to Gel Pak to guarantee stability; f-5, SpotBot tracker; f-6, multiuse TempTale Ultra temperature trackers; f-7, an additional layer of insulation [we use camping gear such as sleeping bag] between the Portable 37°C incubator and the shipping crate to (a) eliminate bouncing during shipment and (b) critically preserve battery charge on an especially cold day; f-8, additional padding, protecting the housing and the temperature indicator against mechanical damage). (g) Geomap (Google Maps) built by SpotBot and mapping (live) movement of the package (except for when it is on the plane; connecting it to the plane Wi-Fi may provide fluent updates of movement and shipment conditions during the flight, but we have not tried it). (h) Impact map during shipment (g-forces acting on the package in *x*-*y*-*z* orientation). The SpotBot will alert the receiving side if an unusually high g-force was experienced by the shipper during transportation (e.g., if it was dropped). (i) Typical TempTale Ultra temperature profile, downloaded after shipment. (j) Retinal organoids (line H1 = WA01 shipped inside portable 37°C incubator)

hPSCs have clusters of cells of all shapes and sizes, in addition to single cells growing in a dense monolayer and with tight adhesion to each other (Hambright et al., 2012; Nasonkin et al., 2009; Nasonkin & Koliatsos, 2006; Singh et al., 2015). Strong forces (e.g., when a box is dropped) during shipment may dislodge clumps of cells and sometimes even part of a monolayer (our observation). For organoids, lateral forces are helpful and move organoids around in the horizontal direction (when a tube is placed horizontally, Figure 1f), enabling aeration and preventing adhesion to each other. When a tube with organoids is shipped in a vertical position, organoids aggregate at the bottom and frequently stick to each other (likely because of the expression of neural cell adhesion molecule, typical for all developing neural cells), which causes problems during transplantation and loading of cannula/transplantation device (Singh et al., 2019). Using the SpotBot device, we were able to pinpoint one location where the package was subjected to excessive g-forces repeatedly and address this issue. The TempTale Ultra temperature trackers serve as an auxiliary temperature recorders and provide a detailed temperature plot (Figure 1i) but only after downloading data from them (not live). For all shipments, we place the shipping container in the same shipping crate. The latter meets FedEx requirements and is pretested (i.e., accepted) by FedEx for shipment by air. The crate bears several shipping indicator stickers: "UN3373" (Category B biological substance), "do not X-ray," and "up" arrows (to emphasize package orientation). The UN3373 sticker is required under the International Air Transport Association shipping guidelines to meet federal shipping regulations (Figure 1a). The shipper is placed inside the crate and further insulated from cold temperatures (we use insulation used in camping gear, which works great) and mechanical stress (bubble wrap) during shipment (Figure 1e), which extends battery life in winter. The housing, which includes a temperature reader (Figure 1f-1) and the fan (Figure 1f-2), is sensitive to mechanical forces and is a critical part of the shipper, necessary for temperature control and circulation during shipment.

3 | ORGANOID PREPARATION FOR SHIPPING

1. Saturate 15 ml of retinal organoid medium (Singh et al., 2019) in a 5% CO_2 incubator for 1 hr using a 100-mm tissue culture dish (rather than 15-ml tube) to maximize saturation of media in the minimum amount of time (normoxia conditions were used, but percentage of oxygen inside the tissue culture incubator may vary; therefore, it is best to saturate media with percentage of oxygen and CO_2 used for the organoid culture).
2. Transfer saturated media into 15-ml tissue culture tube, add organoids, add basic fibroblast growth factor (bFGF) and brain-derived neurotrophic factor (BDNF; 20 ng/ml each, Peprotech, Rocky Hills, NJ, USA, or R&D Systems, Minneapolis, MN, USA), close the cap, seal with parafilm (with the sterile side facing the tube), put a layer of prewarmed heat-absorbing pouches (Cameron's Hot/Cold Gel Pak™, Figure 1f-3), and place the 15-ml tube with organoids horizontally into the shipper (Figure 1f-4).
3. Place the SpotBot tracker (Figure 1f-5) and one of the TempTale Ultra trackers (Figure 1f-6) inside and activate them according to manufacturer's instructions.
4. Place the other TempTale Ultra tracker outside the shipper (Figure 1f-6) in the blue bin and activate.
5. Cover with a cloth/insulation material, enabling easy temperature circulation within the shipper and preventing the tube from bouncing in the shipper.
6. Insulate the shipper inside the crate (Figure 1f-7) and especially the housing with robust layer of insulation (Figure 1f-8) to reduce mechanical damage and battery drain.
7. Remove retinal organoids (shaking slowly inside 37°C tissue culture incubator) at about 1 hr before the nearby FedEx shipping facility closes and ship by "priority overnight" service for delivery by 10:30 a.m. the next day (which is usually 9:00–9:30 a.m. the next day local time). We experimented with 7:30 a.m. delivery the next day (which is an option too) but found it unnecessary. The movement of the package can be monitored from a smart phone or desktop computer using SpotBot software, Figure 1g), unless the package is on the FedEx plane (in the air). This protocol has been successfully tested and used for over 2 years for shipping retinal organoids (Figure 1j) to two surgical sites at Michigan State University (East Lansing, MI, USA, including the coldest times in winter) and University of California Irvine (Irvine, CA, USA) without any impact on organoid viability (Figure 2) and also retinal progenitors dissociated from retinal organoids (to MSU, UTHSCSA, San Antonio, TX, USA, and EYECRO, Oklahoma City, OK, USA).

4 | VIABILITY OF TISSUE AND CELLS AFTER SHIPMENT

Each time, the cultures on arrival were 100% viable (judging by trypan blue staining), and the cells attached to the flasks. When shipping cells in flasks, it is essential to fill the flasks completely with media and seal the caps with sterile parafilm. SYTOX® green (ThermoFisher, Waltham, MA, USA) staining works very accurately for retinal progenitors dissociated from retinal organoids (as a quick assay for evaluating the viability). As for retinal organoids, SYTOX® green staining provides a good pilot estimate of organoid viability (after 30-min staining procedure, according to manufacturer's instructions) but not the absolute numbers of dead cells, due to incomplete penetration into the core of the organoid. For exact estimates of the number of dead cells, we routinely use immunohistochemistry.

To demonstrate the viability of hPSC-derived retinal tissue (organoids) following this shipping protocol, we evaluated cell death in retinal organoids before and after overnight shipping by (a) immunohistochemistry with cleaved Caspase-3 (Cell Signaling, Cat. #9664, Figure 2, middle panel) and (b) SYTOX® green staining (Figure 2, bottom panel). In both cases, we found the number of cells positive for cell death negligible, about 1% or less. To address the consistency of successful survival of retinal organoids during shipment, we processed (with immunohistochemistry to cleaved Capase-

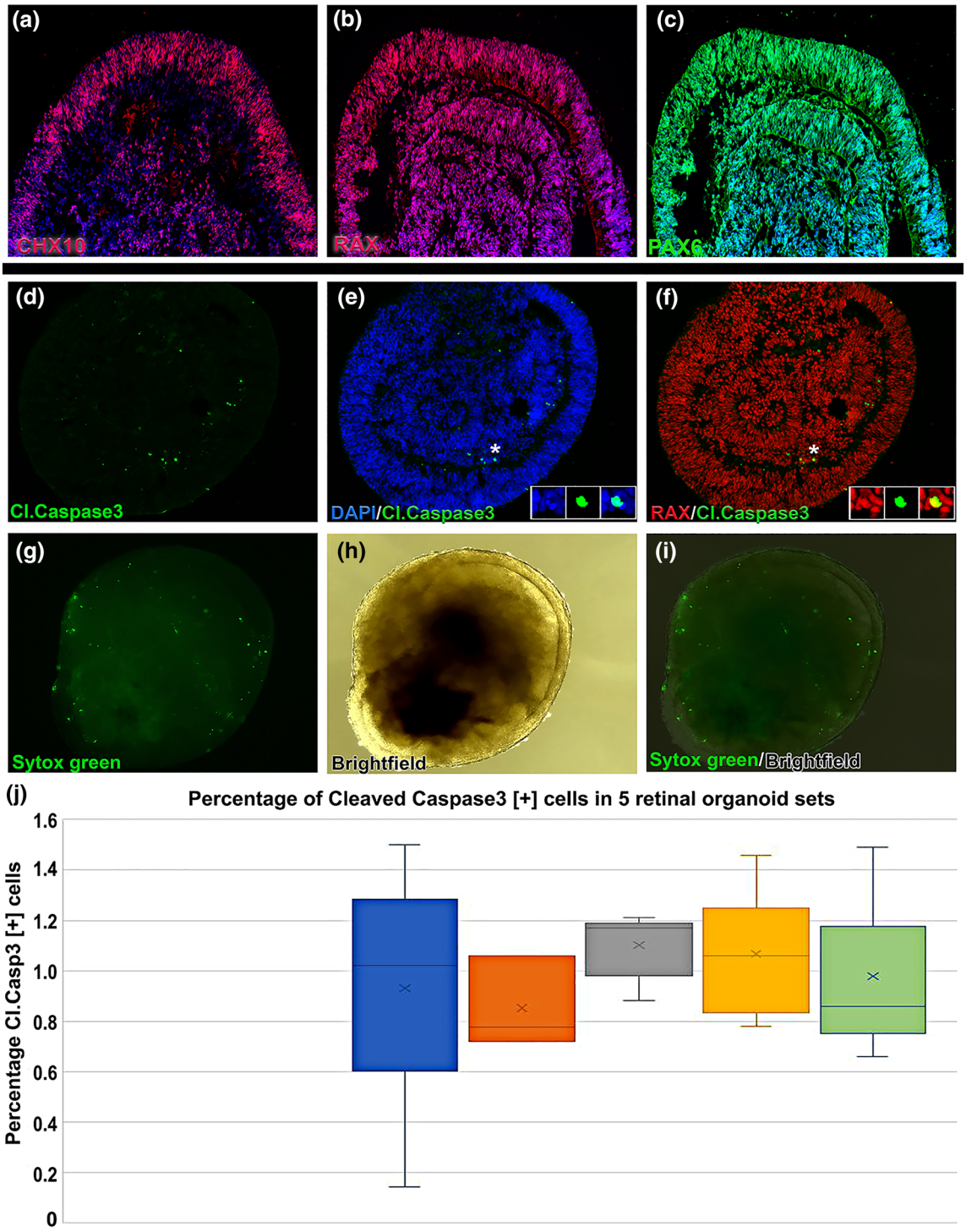

FIGURE 2 Cleaved Caspase-3 and SYTOX® green markers in organoids shipped overnight at 37°C. Cleaved Caspase-3 and SYTOX® green markers in organoids shipped overnight at 37°C. Evaluating cell death in human pluripotent stem cell retinal tissue (retinal organoids) after overnight shipment in our 37°C shipper by two methods: frozen immunohistochemistry with cleaved Caspase-3 (early cell death marker) antibody and SYTOX® green staining. (a–c) Retina-specific staining with antibodies to CHX10, RAX, and PAX6, respectively, and pan-nuclear DAPI counterstain. (d–f) Typical distribution of cleaved Caspase-3 in retinal organoids after overnight shipping procedure (the pattern before and after shipping was identical); insets represent magnifications of areas shown with asterisks in the Panels (e) and (f). (g–i) Evaluating cell death in the cultured whole retinal organoids after shipment with SYTOX® green dead cell stain (impermeant to live cells), 1:10,000 dilution for 30 min: (g) is SYTOX® green only, (h) is a brightfield image and (i) is a merged image (green+brightfield). (j) A box plot showing the distribution of percentages of cell death [+] marker cleaved Caspase-3 in four sets of shipped organoids (orange, gray, yellow, and green boxes) in addition to organoids before shipment (blue box). Very similar percentage of cleaved Caspase-3 was found in all five samples (blue box = organoids before shipping: 0.93%, SEM 0.15; orange, gray, yellow, and green boxes = organoids after shipping, 0.85%, SEM 0.01; 1.1%, SEM 0.06; 1.07%, SEM 0.08; and 0.98%, SEM 0.09, respectively)

3) a total of four sets of organoid samples shipped to surgical sites. These were unused (leftover) organoids, which were fixed by the surgical team and shipped to us after surgeries. We used the box plot (Figure 2j) to visually show the distribution of percentages of cell death [+] marker cleaved Caspase-3 in each set. We see very similar percentage of cleaved Caspase-3 in all five samples (blue box = organoids before shipping: 0.93%, SEM 0.15; orange, gray, yellow, and green boxes = organoids after shipping, 0.85%, SEM 0.01; 1.1%, SEM 0.06; 1.07%, SEM 0.08; and 0.98%, SEM 0.09, respectively). The analysis of variance (ANOVA) showed no statistically significant difference in the number of cleaved Caspase-3 [+] cells in all five samples. An additional observation, which we routinely used for evaluating the successful delivery of retinal organoids, was (a) lack of cell debris in the culture and (b) survival of leftover organoids (left after surgeries) in tissue culture for a week or more and, importantly, successful in vivo engraftment in subretinal space. The number of cleaved Caspase-3 [+] cells in retinal organoids (before and after shipment) was similar to those reported in an earlier study that generated retinal organoids (Zhong et al., 2014). This clearly demonstrates that the shipping protocol we have developed robustly maintains the viability of the hPSC-derived retinal tissue. Furthermore, we report that the SYTOX® green method can be efficiently used to quickly evaluate the viability of retinal organoids after shipping and ahead of surgery (by sampling the subset of organoids, assigned for testing and not surgery with 30-min SYTOX® green assay). This, in turn, ensures that retinal tissue from organoids subjected to harsh shipping conditions is identified and not used for implantation into animals, thus streamlining in vivo work and guaranteeing better and more reproducible results. We recommend testing SYTOX® green on the whole retinal organoids and following the recommended dilutions provided by the manufacturer (1:10,000–1:30,000). As a positive control, precut retinal organoids, which have spent several hours outside of the tissue culture incubator, repeatedly displayed evidence of excessive cell death when stained with SYTOX® green.

Developing preclinical vision restoration protocols based on transplanting stem cell–derived retinal tissue (Lin et al., 2018; Singh et al., 2019) or cells (Hambright et al., 2012) into the ocular space (subretinal or epiretinal) of experimental animals requires close coordination of work between the team generating the organoids and the team doing surgical grafting. Precise and reliable methods are needed, because (a) biological material is expensive and takes time to generate, (b) the surgical team, equipment, and surgical rooms are scheduled in advance and availability of all three takes time to plan, and (c) critically, the animals, especially those models with progressive retinal atrophy (Petersen-Jones & Komaromy, 2015; Seiler et al., 2014), are scheduled for surgeries on specific days depending on the dynamics of retina degeneration and multiple experiments are usually done before each study is completed (with each cohort of animals being implanted at the same age, to be able to pool the data from multiple experiments). Developing neural retinal tissue is sensitive to shipping conditions and needs to be transported quickly and in the optimal conditions to maintain viability ahead of transplantation. Therefore, if hPSC retinal tissue (organoids) arrives damaged, this is costly and impacts the project. This is especially true with large animal models, and one failed experiment may delay results for the next 3–6 months (due to breeding constraints). To address this logistical problem in preclinical translational work, we reviewed all available options for overnight long-distance shipping of viable hPSC retinal tissue for transplantation and developed and refined this protocol, thus enabling predictable, cost-effective, and reliable delivery of viable hPSC retinal tissue. Optimal tissue viability is essential for the overall reproducibility and success of the in vivo work. This method works equally well for shipping hPSC retinal tissue and cultured neural retinal progenitors. It will be applicable for teams doing in vitro and in vivo work at different locations, for precise coordination of tissue shipping, receiving, and implantation.

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CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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