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Noninvasive high-resolution *in vivo* imaging of cell biology in the anterior chamber of the mouse eye

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Abstract

There is clearly a demand for an experimental platform that enables cell biology to be studied in intact vascularized and innervated tissue *in vivo*. This platform should allow observations of cells noninvasively and longitudinally at single-cell resolution. For this purpose, we use the anterior chamber of the mouse eye in combination with laser scanning microscopy (LSM). Tissue transplanted to the anterior chamber of the eye is rapidly vascularized, innervated and regains function. After transplantation, LSM through the cornea allows repetitive and noninvasive *in vivo* imaging at cellular resolution. Morphology, vascularization, cell function and cell survival are monitored longitudinally using fluorescent proteins and dyes. We have used this system to study pancreatic islets, but the platform can easily be adapted for studying a variety of tissues and additional biological parameters. Transplantation to the anterior chamber of the eye takes 25 min, and *in vivo* imaging 1–5 h, depending on the features monitored.

Introduction

Fundamental understanding of cellular processes in health and disease has been gained by studying cells of various tissues *in vitro*. However, results obtained from experiments *in vitro* are often insufficient to explain the performance of cells in more physiological settings like whole organs or living organisms. To place observations made in an *in vitro* system into a physiological context, studies have to be performed under *in vivo* conditions.

In vivo imaging methods

Noninvasive *in vivo* imaging techniques such as computer tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET) or bioluminescence imaging (BLI) have been increasingly used to investigate cell and organ function in small animals *in vivo*. Emerging advances in equipment and probe engineering over recent years has enabled functional and molecular imaging using these techniques^{1,2}. Choosing the appropriate modality depends on the general characteristics of these techniques, which differ in properties such as penetration depth, spatial and temporal resolution, sensitivity and

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availability of traceable probes. One major advantage of these techniques is their penetration depth, which is only in the case of BLI limited to 1–2 cm and has no limit in CT, MRI and PET². However, a limitation to all these techniques for the study of cellular events is their spatial resolution and/or sensitivity^{1,2}. Although MRI has the highest resolution of 25–100 mm, its sensitivity is comparably low, with 10^{-3} – 10^{-5} mole/L. PET has a reasonably high sensitivity of 10^{-11} – 10^{-12} mole/L, but, with a resolution of 1–2 mm, information obtained with this technique always reflects a relatively large number of cells^{2,3}.

In contrast, confocal and two-photon laser scanning microscopy (TPLSM) provide subcellular resolution and easily enable assessment of cellular events, interactions of neighboring cells and morphology at a micrometer scale. Moreover, acquisition speed and the potential to image multiple parameters simultaneously using several fluorophores are major advantages when investigating cellular mechanisms³. Application of LSM for *in vivo* studies is usually limited due to a relatively restricted working distance and imaging depth, which, depending on the studied tissue, is maximally 1 mm (refs. 3,4). Accessing target cells for the application of LSM is mostly invasive and often excludes the possibility of repetitive examinations. Therefore, *in vivo* studies utilizing LSM have, until recently, only been applied to a limited number of relatively easily accessible organs.

High-resolution *in vivo* imaging in the anterior chamber of the eye

Here we provide a step-by-step protocol for noninvasive longitudinal *in vivo* studies of cell biology at single-cell resolution, utilizing LSM and taking advantage of the cornea as a natural body window. For this purpose, the tissue of interest is transplanted into the anterior chamber of the eye and cell biological parameters are assessed by LSM through the cornea.

The anterior chamber of the eye has been frequently used as a transplantation site to study a variety of tissues^{5–9}. Although originally the anterior chamber of the eye was selected as a transplantation site because of its properties as an immune privileged site¹⁰, most studies use this location in a syngeneic transplantation setting, because it is easily accessible and the cornea allows macroscopic observation of the engrafted tissue. Additionally, the iris, which forms the base of the anterior chamber, has one of the highest concentrations of blood vessels and autonomic nerves in the body, and thereby enables fast reinnervation¹¹ and revascularization¹² of the graft. To date, studies utilizing the anterior chamber of the eye as a transplantation site mainly used macroscopic observations⁷ to investigate graft physiology. This restricts longitudinal studies to parameters observable at low resolution. *In vivo* electrophysiology⁹ as well as histology⁵ and various other *in vitro* techniques after graft removal enable assessment of morphology and cellular function. However graft removal sets an endpoint to the study and thereby prevents longitudinal monitoring. We now combine transplantation of tissue to the anterior chamber with high-resolution LSM to enable longitudinal noninvasive *in vivo* studies of cell biology at a subcellular level.

We have recently used the protocol described in this article to transplant isolated pancreatic islets of Langerhans into the anterior chamber of the eye¹³. Studies on vascularized and innervated islets of Langerhans are necessary to understand the mechanisms leading to hormone secretion in health and in diseases such as type 1 and type 2 diabetes. However, due to the scattered distribution of islets of Langerhans throughout the exocrine pancreas and the anatomy of the rodent pancreas in particular, noninvasive longitudinal *in vivo* studies of the islets of Langerhans at single-cell resolution were not previously feasible. Techniques used to study the biology of islet of Langerhans *in situ*, using LSM, were rather invasive and did not allow repetitive imaging at all or only over a limited time period^{14,15}. We showed that, after transplantation to the anterior chamber of the eye, isolated islets readily engrafted¹³. Diabetic mice could be rendered normoglycemic by transplanting islets of Langerhans to the anterior chamber of their eye, and these mice showed identical

responses to glucose tolerance tests compared to control mice. We were able to longitudinally monitor morphology of the islets and to follow the revascularization process. We could also repetitively measure systemically induced changes in cytoplasmic free Ca^{2+} concentration in β cells of the same islet. Finally, we noninvasively monitored chemically induced cell death in islets after systemic injection of a β cell toxin¹³.

Advantages and applications of the system

Our platform enables noninvasive assessment of multiple morphological and functional parameters in vascularized and innervated tissue, thus making it possible to study complex biological interactions in an *in vivo* system at single-cell resolution. Although our study was performed on pancreatic islets of Langerhans, the platform can easily be used to investigate a variety of tissues. Different types of tissues, transplanted to the anterior chamber of the eye, have been shown to establish organotypic vascularization^{12,16} and innervation^{6,7,17}. This allows these tissues to be studied in a setting comparable to their natural surrounding without having to access the tissue in an invasive manner. Moreover, our protocol is not limited to the use of mice as donors or recipients. Studies similar to those described here can also be performed using rats as recipients (S.S., D.N., M.K., I.B.L. and P.-O.B., unpublished data). Additionally, using immune-deficient mice as recipients allows not only allogeneic, but also xenogeneic transplantations, for example, human islets of Langerhans transplanted to nude mice (A.C. and P.-O.B., unpublished data). Implementation of newly developed fluorescent proteins, biosensors and countless available transgenic animals expressing fluorescent markers, lacking specific proteins or resembling complex diseases will facilitate investigations of numerous parameters important for development, function and survival of transplanted cells under physiological and pathophysiological conditions. Furthermore, due to the anatomical characteristics of the eye, the target cells and their regulatory input can be modulated not only systemically but also locally without difficulty. Substances can be applied topically onto the eye or injected into the anterior chamber. Additionally, perfusion of the anterior chamber repetitively allows exchange of the aqueous humor or loading of the graft with fluorescent indicators.

Limitations of the system

Special anatomical and functional characteristics of the anterior chamber of the eye, required to optimize vision and to create an immune privileged site, have to be taken into consideration when using it as a transplantation site. Differences in the composition of the aqueous humor inside the anterior chamber of the eye and the blood plasma have to be kept in mind when interpreting observations made at this location. However, so far our studies have not shown obvious effects on normal graft function by the local environment¹³ (S.S., D.N., M.K., A.C., I.B.L. and P.-O.B., unpublished data). One possible explanation might be that the induced neovascularization leads to the presence of a different vascular morphology compared to the morphology of the iris vascularization, as the vascular morphology is determined by the grafted tissue^{12,16}. In the case of engraftment of islets of Langerhans, this leads to the presence of fenestrated endothelial cells¹². The resulting break in the blood–aqueous barrier allows for exchange of aqueous humor and blood plasma and therefore minimizes differences in their composition.

Experimental design

Choice of donor and recipient mice

The choice of donor and recipient mice is important and depends on the aim and design of the study. If immunological responses need to be avoided, syngeneic transplantations should be performed, if possible. Otherwise, immune-deficient mice, for example, nude mice, can be used to avoid an immune response in allogeneic or xenogeneic transplantation settings.

The use of albino mice facilitates some studies due to the unpigmented iris, but is not essential.

Furthermore, the choice of transgenic animals expressing fluorescent proteins or biosensors regulated by specific promoters determines the assessable parameters and imaging settings. For example, a fluorescent protein, such as GFP, expressed using the rat insulin promoter (RIP) allows visualization of β cells; expression controlled by the Tie2 promoter allows detection of endothelial cells. If fluorescent proteins are to be combined in an experiment with additional fluorescent indicators (e.g., systemically injected fluorescent dextrans to visualize the vascular system), their respective excitation and emission spectra must be taken into account and must, of course, be appropriate for the imaging setup (excitation source and emission detection).

Time range of engraftment

Depending on the study parameters, it may be necessary to evaluate the time of tissue engraftment before defining experimental time points. If studies are to be performed on fully engrafted tissue, revascularization and reinnervation have to be finalized. Time periods may vary between different species, strains and pretransplantation conditions. Also the time period between revascularization and reinnervation might differ significantly. If engraftment should be assessed, reasonable time periods between imaging should be chosen. At certain periods of engraftment (e.g., shortly after transplantation), changes in morphology and function might be more dramatic than at others, and therefore imaging will need to be done more frequently.

Choice of anesthetic

When choosing the anesthetic for the experiments, several parameters have to be taken into account. Optimal anesthetics have short recovery periods and minimal side effects on the animal. However, anesthetics might also affect the parameter investigated during the study; functional parameters in particular can be influenced by certain compounds used for anesthesia. Therefore, all possible effects of the anesthesia have to be carefully evaluated to avoid false interpretations of the obtained results.

Materials

Reagents

- Donor and recipient mice have to be chosen according to the aim and design of the study (see Experimental design). To achieve the results presented here we used: NMRI mice (Charles River Laboratories) or (Scanbur); Tie2-GFP mice (Strain Tg(TIE2GFP)287Sato/J; Jackson Laboratory); transgenic mice with fluorescent reporters expressed in pancreatic β cells under the insulin promoter (RIP-GFP). Alternatively, (Strain B6.Cg-Tg(Ins1-DsRed*T4)32Hara/J) or (Strain B6.Cg-Tg(Ins1-EGFP)1Hara/J) (Jackson Laboratory) can be used for visualization of β cells

! CAUTION All animal studies must conform to national and institutional regulations. This protocol was approved by the local animal ethics committees at Karolinska Institutet (Local Animal Ethics Committee of Karolinska Institutet) and the University of Miami (University of Miami Animal Care and Use Committee).

Isoflurane (Abbott, cat. no. B506 or Baxter, cat. no. KDG5623)

- 40% O₂ in 60% N₂ (AGA)
- Buprenorphine (Reckitt Benckiser Pharmaceuticals or Schering-Plough)

- Viscotears (Novartis)
- Alloxan (Sigma, cat. no. A7413)
- Texas Red 70-kDa dextran (Invitrogen, cat. no. D1830)
- Annexin VAPC (Invitrogen)
- Hypnorm (VetaPharm)
- Dormicum (Roche)
- Sterile water for injection (Braun, cat. no. 3526259)
- Fluo-4 AM special packaging (Invitrogen, cat. no. F14201)
- Fura-Red AM special packaging (Invitrogen, cat. no. F3021)
- Pluronic F-127 (Invitrogen, cat. no. P3000MP)
- Glybenclamide (Sigma, cat. no. G-0639)
- Extracellular solution (see REAGENT SETUP)

Equipment

- $27\text{ G} \times \frac{3}{4}\text{''}$ Nr. 20, $0.4 \times 19\text{ mm}^2$ Microlance (BD, cat. no. 302200)
- Blunt 27-G cannula, custom made of a 27-G needle
- 0.5-ml Threaded plunger Hamilton gastight syringe no. 1750 (Hamilton, cat. no. 81242)
- Portex tubing polyethylene 0.4 mm internal diameter (i.d.), 0.8 mm outer diameter (o.d.) (Scientific Labs, cat. no. 800/100/140)
- Polyethylene tubing 0.28 mm i.d., 0.61 mm o.d. (Scientific Labs, cat. no. 800/100/100)
- Polyethylene tubing 0.86 mm i.d., 1.27 mm o.d. (Scientific Labs, cat. no. 800/100/260)
- Tygon tubing 0.76 mm i.d., 0.86 mm wall thickness (Ismatec, cat. no. 070535-081)
- 1-ml Syringe Omnitix (Braun, cat. no. 9161406V)
- 5- and 10-ml Syringes Plastipak (BD, cat. no. 302187 and 302188)
- 50-ml Reagent tube (Sarstedt, cat. no. 62.547.254) 400 Anesthesia Unit (Univentor)
- 400 Anesthesia Unit (Univentor)
- Microsyringe no. 705, 0.5 ml (Hamilton)
- Stereomicroscope MZ FLIII (Leica).
- Head-holding adapter (SG-4N-S; Narishige, cat. no. SG-4N-S)
- Gas mask (GM-4-S; Narishige, cat. no. GM-4-S)
- UST-2 Solid Universal Joint (Narishige, cat. no. UST-2)
- Dumont no. 5 forceps (Fine Science Tools, cat. no. 11251-10)
- Custom-made heating pad

- DMLFSA upright microscope, equipped with a TCS-SP2-AOBS confocal scanner (Leica)
- Ti:Sapphire laser Tsunami (Spectra-Physics)
- 2.5× and 5× objectives (Leica, cat. nos. 506083 and 506504)
- Long distance water-dipping lenses (Leica HXC APO 10 × 0.3 W, 20 × 0.5 W, 40 × 0.8 W; Leica, cat. nos. 506142, 506147 and 506155)
- Micromanipulator 5171 (2) (Eppendorf)
- Universal capillary holder (2) (Eppendorf, cat. no. 5242 150. 009)
- Capillary grip head 1 (2) (Eppendorf, cat. no. 5242 023. 007)
- Thin-wall borosilicate glass capillaries without filament TW120-4 (WPI, cat. no. TW120-4)
- DMZ universal puller (Zeitz Instrumente)
- Micropipette beveler BV-10 (Sutter Instruments, cat. no. BV-10)
- 802 Syringe pump (Univentor)
- Leica Confocal Software (version 2.61; Leica) Volocity (Improvision)
- Volocity (Improvision)
- Matlab (The Math Works)
- Wavelet filtering algorithm¹⁸

Reagent Setup

Extracellular solution 140 mM NaCl, 5 mM KCl, 2 mM NaHCO₃, 1 mM NaH₂PO₄, 1.2 mM MgCl₂, 2.5 mM CaCl₂, 10 mM HEPES, 3 mM glucose (pH 7.4 with NaOH).

Equipment Setup

Confocal and two-photon setup For LSM, we use a Leica TCS-SP2-AOBS (acousto optical beam splitter) confocal laser scanner equipped with Argon and HeNe lasers connected to a Leica DMLFSA microscope. Two-photon excitation is achieved using a Ti:Sapphire laser (Tsunami; Spectra-Physics) for ~100 fs excitation at ~82 MHz. The microscope stage is customized for the use of a head-holding adaptor and mouse (Fig. 1).

Head-holding adapter To fix the head of the mouse for surgery and imaging, we use a head-holding adapter (Fig. 2a). Depending on the type of anesthesia used, the head holder is equipped with a nosepiece (Fig. 2b) or a gas mask (Fig. 2c). The head holder (Fig. 1(3)) is attached to a metal plate (Fig. 1(2)), which fits onto the customized stage of the microscope (Fig. 1(1)). The metal plate is covered by a heating pad (Fig. 1(5)). Body temperature is controlled via a rectal probe, which regulates the temperature of the heating pad.

Eye stabilizer For retraction of the eyelids and additional stabilization of the eye we use a custom-made supporting device (Figs. 1(4), 2a and 3). Attach a no. 5 Dumont forceps to a small metal bar and clamp the metal bar into a UST-2 Solid Universal Joint. Fix the Universal Joint to the same metal plate as the head holder on a level with the eye, so you can reach the eyes with the tips of the forceps. Cover the tips of the forceps with a piece of polyethylene tube, creating a loop between the tips. At the front of the forceps, attach a screw to enable adjustment of the distance between the forceps tips (Fig. 3).

Islet injection Connect ~10 cm of 0.4 mm i.d. polyethylene tubing with a 27-G needle to the 0.5-ml Hamilton syringe and insert the blunt 27-G cannula at the other end of the tubing. During injection into the anterior chamber, fix the Hamilton syringe to the table to allow the cannula to be held with one hand while injecting with the other (only possible if using the head holder for transplantation).

Custom-made gas mask For transplantation without using the head holder prepare a small gas mask from a 5-ml plastic syringe by removing the piston and cutting the syringe ~1 cm above the bottom. Connect the tubing of the anesthetic pump to the needle fitting.

Anterior chamber perfusion *Outflow*: connect a ~35-cm piece of polyethylene tubing 0.86 mm i.d. and 1.27 mm o.d. with one end to a capillary holder and connect the other end of the tubing to a 10-ml syringe without a plunger (open reservoir). *Inflow*: connect a ~35-cm piece of tygon tubing 0.76 mm i.d. and 0.86 mm wall thickness with one end to a capillary holder and connect the other end of the tubing to a 1-ml syringe.

Micropipette preparation Pull pipettes for perfusion from glass capillaries using a regular pulling program for patch pipettes. Break the pipette to a tip diameter of 30–40 μm . Bevel the tips at an angle of 35° to a final diameter of 70–90 μm . Pipettes for the outflow should be slightly bigger (~90 μm) than pipettes for the inflow (~70 μm).

Image processing To denoise images captured with confocal LSM and TPLSM we use wavelet filtering¹⁹. For analysis and image display, we use processing software Volocity and Leica confocal software.

Procedure

Transplantation of pancreatic islets to the anterior chamber of the eye

1. Isolate mouse pancreatic islets as previously described^{20,21}. Culture islets as required, depending on study parameters (see INTRODUCTION).
2. Transfer 30–40 islets from the culture medium to a dish containing sterile PBS using a pipette and center the islets in a cluster as compact as possible in the middle of the dish by rotating the dish in small circles.
3. Aspirate the islets into the blunt 27-G cannula and the connected polyethylene tubing. The islets should be aspirated in a minimal volume (10–20 μl) to facilitate injection into the anterior chamber. To further decrease the injected volume, let the islets sink to the end of the tube toward the cannula by hanging the tube upside down until injection.

▲ **Critical Step** Aspirating the islets into too large a volume will lead to difficulties during the injection process. It will expose the eye to unnecessarily high intraocular pressure and may result in reflux of islets out of the anterior chamber after removing the cannula.
4. Put a piece of cotton wool in a 50-ml reagent tube and drop ~ 1 ml isoflurane onto the wool. Stun the mouse by holding the head into the reagent tube for a few seconds.
5. Place the mouse in the head holder (see EQUIPMENT SETUP) under a stereomicroscope and fix the head with the eye selected for transplantation facing upward. If not using a head holder, place the mouse under a stereomicroscope on a heating pad with the eye selected for transplantation facing upward and put the nose of the mouse in the gas mask (see EQUIPMENT SETUP).

6. Anesthetize the mouse using isoflurane, 2–2.5% (vol/vol) in 40% (vol/vol) O₂ and 60% (vol/vol) N₂.
! Caution Isoflurane levels must be carefully controlled to ensure a proper state of anesthesia, by checking reflexes and physiological parameters such as breathing or heart rate. Vacuum is applied in the area of anesthesia to protect the operator.
7. Inject buprenorphine (0.05–0.1 mg kg^{−1}) subcutaneously to relieve postoperative pain.
8. If using a head holder, carefully pull back the eyelids and gently place the polyethylene tubing loop of the eye stabilizer (see EQUIPMENT SETUP) below the corneoscleral junction. Otherwise, use one hand to retract the skin around the eye to visualize the corneoscleral junction of the eye and gently fix the position of the head. Be careful not to interrupt the breathing or blood circulation while holding the mouse.
▲ Critical Step Great care has to be taken when placing the stabilizing forceps. Pay attention not to disrupt blood circulation in the eye. This can be easily assessed by observing the movement of erythrocytes in the blood vessels of iris and graft.
9. Connect a 27-G needle to a 1-ml syringe to ease handling. Use the 27-G needle to puncture the cornea close to the sclera while taking great care not to damage the iris and to avoid bleeding.
10. Gently insert the blunt cannula into the anterior chamber of the eye through the hole made with the needle. Slowly inject the islets into the anterior chamber. After injection, carefully withdraw the cannula.
11. Leave the anesthetized mouse in the head holder for an additional 10–15 min. In our experience, a short delay after transplantation before waking up the animal is sufficient to facilitate engraftment of the tissue at the position it settled after injection.
12. Put a drop of Viscotears on the eye to prevent desiccation.
13. Remove the mouse from the head holder, turn off the isoflurane and observe the mouse to ensure full recovery before returning it to its cage.
14. Return the mouse to its cage until the determined time point to image the transplanted islets. The length of time required will depend on the parameters of the experiment (see Experimental design).

Imaging of islets engrafted in the anterior chamber of the eye

When ready to image the transplanted cells, stun the recipient mouse, anaesthetize and expose the eye containing the transplanted islets as described in Steps 5–6 and 8–9. For imaging, head holder and eye stabilizer should be used. If buprenorphine is injected (as in Step 7) this should be done ~20 min before the end of the experiment.

Place the head holder together with the mouse under an upright microscope equipped for confocal LSM and TPLSM.

To get an overview of the eye, use low-magnification objectives (2.5 and 5×). For high-resolution LSM, use water immersion dipping objectives (10, 20 and 40×) with a long-working distance using filtered saline or Viscotears as immersion liquid between the lens and the cornea.

▲ Critical Step Apply the minimum required laser power and scan time necessary for visualization to avoid photodamage and bleaching.

? Troubleshooting

Image the biological parameter of interest, as detailed in the appropriate option below:

A. Imaging graft morphology

- i. To visualize cell-specific morphology, islets of transgenic mice expressing a fluorescent protein in β cells (e.g., RIP-GFP) can be used for transplantation. Excite GFP fluorescence with a 488-nm laser and detect emission between 495 and 530 nm. Islet morphology can also be imaged by detection of a reflection image. Choose a laser (e.g., 633 nm) and set the AOBS control to optimize reflection detection. Collect emission at ± 4 nm of the laser wavelength.

B. Imaging vascularization in the iris and the engrafted islets

- i. Vascularization can be visualized by imaging GFP-expressing endothelial cells (e.g., from Tie2-GFP donor mice; excite GFP fluorescence with a 488-nm laser and detect emission between 495 and 530 nm) or, alternatively, by imaging the blood vessel lumen using fluorescently labeled dextrans, as described below.
- ii. Inject 0.1 ml of 10 mg ml⁻¹ of a fluorescently labeled dextran (70 kDa) intravenously into the tail vein.
- iii. Following injection of a fluorescently labeled dextran, image the engrafted islets using appropriate settings for the chosen dextran. For simultaneous imaging of β cells and vessels, transplant islets from RIP-GFP mice and inject Texas-Red conjugated dextran (70 kDa). Excite Texas Red and GFP with a two-photon laser at 890 nm and collect emission light onto nondescanned detectors using a dichroic mirror (RSP 560) and emission filters (BP 525/50 and BP 640/20).

? Troubleshooting

C. Imaging β cell death

- i. Induce β cell death in the engrafted islets by intravenous injection of alloxan (75 mg per kg body weight).
■ Pause Point Wait for 24 h for alloxan to induce β cell death.
- ii. Measure blood glucose levels 24 h after the administration of alloxan to confirm that the mouse has been rendered hyperglycemic.
- iii. Inject 0.1 ml of annexin V-APC intravenously via the tail vein. Wait for 4–6 h for annexin V-APC to label apoptotic and dead cells.
- iv. Image β cell death in the engrafted islets between 4 and 6 h following the administration of annexin V-APC, using appropriate settings for allophycocyanin (APC) fluorescence. Excite APC at 633 nm with collection of emission light between 645 and 680 nm.

D. Imaging cytoplasmic free Ca²⁺ concentration after loading the graft with Ca²⁺ indicators via perfusion of the anterior chamber of the eye

- i. The protocol described here for perfusion of the anterior chamber of the eye (see Fig. 4) is modified from ref. 22. Fill the syringes, tubing and capillary holders with filtered extracellular solution and place the pipettes (see EQUIPMENT SETUP) in the capillary grip head of the capillary holder.
- ii. Anesthetize the mouse by an intraperitoneal injection of 100 μ l per 10 g bodyweight of a Hypnorm/sterile water/Dormicum mix (1:2:1). Anesthesia will set in within 1–2 min. Prolong anesthesia by injections of 50 μ l per 10 g bodyweight of a Hypnorm/sterile water mix (1:3) after 30 and 60 min. If necessary, prolong anesthesia further after 90 min by an injection of 50 μ l per 10 g bodyweight of the initial Hypnorm/sterile water/Dormicum mix (1:2:1).

▲ **Critical Step** Care has to be taken when choosing anesthetic for functional studies on islet cells, as several compounds have been reported to exert an effect on blood glucose levels and insulin secretion^{23,24}. Isoflurane has been shown to inhibit glucose stimulated insulin release by a direct mechanism on islet cells, and therefore is not suitable for functional studies²⁵. A mix of Hypnorm/Dormicum does not seem to interfere with the measurements of changes in cytoplasmic free Ca^{2+} concentration.
- iii. Put the mouse in the head holder and fix the head with the eye containing the transplanted islets facing upward.
- iv. Carefully pull back the eyelids and gently place the polyethylene tubing loop of the eye stabilizer below the corneoscleral junction and place holder and mouse under the upright microscope.

▲ **Critical Step** Great care has to be taken when placing the stabilizing forceps. Pay attention not to disrupt blood circulation in the eye.
- v. Hang the open reservoir of the outflow at a height of ~21 cm above the eye to ensure a constant intraocular pressure of ~15 mm Hg. Place the capillary holder of the outflow in the micromanipulator.
- vi. While using a 2.5 $\times$ objective to observe the entire eye, insert the outflow pipette into the anterior chamber with the micromanipulator. The pipette should penetrate swiftly through the cornea at an angle of ~15°.

▲ **Critical Step** Be careful not to unnecessarily scratch the cornea or to damage the iris.
- vii. Aspirate ~130 μ l of the Fluo-4/Fura-Red mix (1:1, 500 μ M each) into the inflow capillary and fix the capillary holder onto the micromanipulator. Make sure that there is enough dye-free extracellular solution in the syringe for wash out. Place the 1-ml syringe of the inflow into the syringe pump.
- viii. Insert the inflow pipette into the anterior chamber opposite the outflow pipette. Penetrate the cornea as described in Step 18D(vi).

- ix. Initially, exchange the aqueous humor with the perfusate at a fast rate ($\sim 10 \mu\text{l}$ in 30 s). Observe through the microscope the fluid entering the anterior chamber and exiting through the outflow micropipette. This can be facilitated by using a fluorescent lamp if fluorescent indicators are perfused. Additionally, make sure the anterior chamber is not swelling due to increased intraocular pressure.

? Troubleshooting

- x. Continuously perfuse the anterior chamber of the eye at a rate of $\sim 3 \mu\text{l min}^{-1}$ for ~ 40 min.
- xi. After loading, wash out the dye in the anterior chamber by perfusing the anterior chamber at a fast rate ($\sim 10 \mu\text{l min}^{-1}$).

▲ **Critical Step** During all perfusion steps, regularly control the functionality of the perfusion. Pay attention that the eye is not swelling due to a blocked outflow.

- xii. After washing out the dye, switch off the perfusion. Do not remove the pipettes.
- xiii. For imaging of changes in cytoplasmic free Ca^{2+} concentration, switch to a higher magnification water immersion dipping objective (10, 20 or $40\times$) and apply Viscotears as immersion liquid.
- xiv. Simultaneously image Fluo-4 and Fura-Red to enable ratiometric measurements of changes in cytoplasmic free Ca^{2+} concentration. Excite Fluo-4 and Fura-Red at 488 nm, and collect emission light for Fluo-4 between 495 and 535 nm, and for Fura-Red, between 600 and 700 nm.

▲ **Critical Step** Apply the minimum required laser power and scan time necessary for visualization to avoid photodamage and bleaching.

Start acquiring a time series of the Fluo-4 and Fura-Red fluorescence in the cells of interest.

- xv. After acquiring a baseline of unstimulated fluorescence levels, stimulate systemic insulin release by injecting glybenclamide (1 mg kg^{-1}) intravenously via the tail vein. Changes in cytoplasmic free Ca^{2+} concentration in β cells within the islet graft should be observed within seconds after injection.
 - xvi. After imaging, remove the pipettes carefully from the eye.
 - xvii. To relieve post-operative pain, inject the mouse subcutaneously with buprenorphine ($0.05\text{--}0.1 \text{ mg kg}^{-1}$).
- ▲ **Critical Step** Buprenorphine must not be injected before ending the experiment as it acts as an antidote to the analgetic compound of Hypnorm (Fentanyl).
- xviii. Place the mouse in a warm environment ($\sim 30^\circ\text{C}$) until it wakes up after Hypnorm/Dormicum anesthesia. This can take several hours.

•Timing

Step 1, isolation of islets: ~4 h; dependent on the number of mice used for isolation

Steps 2–14, transplantation of islets to the anterior chamber: ~25 min per mouse

Step 18A, imaging of graft morphology: ~1 h per mouse; dependent on the amount of tissue transplanted

Step 18B, imaging of graft vascularization: ~1 h per mouse; dependent on the amount of tissue transplanted

Steps 18C(i–ii), induction of cell death with alloxan: ~24 h

Steps 18C(iii–iv), labeling of dead and apoptotic cells: ~6 h

Step 18C(iv), imaging of β cell death: ~1 h; dependent on the amount of tissue transplanted

Equipment setup, preparation of micropipettes for anterior chamber perfusion: ~1.5 h per 4–6 pipettes

Steps 18D(i–xix), imaging of cytoplasmic free Ca^{2+} concentration after loading the graft with Ca^{2+} indicators via perfusion of the anterior chamber of the eye: ~2 h per mouse

Anticipated Results

The platform described here enables longitudinal assessment of several biological parameters *in vivo* without the need for invasive surgical procedures to access the tissue of interest. Following transplantation of pancreatic islets of Langerhans to the anterior chamber of the eye, the current protocol easily enables detection of islet graft morphology by reflection or fluorescence imaging (Fig. 5). Furthermore, vascularization (Fig. 6) and cell death (Fig. 7) can be followed longitudinally by systemic injections of fluorescent dyes. Additionally, islet cells can be loaded repetitively with Ca^{2+} indicators, by perfusion of the anterior chamber (Figs. 4 and 8), to measure systemically induced change in cytoplasmic free Ca^{2+} concentration.

? Troubleshooting

Troubleshooting advice can be found in Table 1

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References

1. Koo V, Hamilton PW, Williamson K. Non-invasive *in vivo* imaging in small animal research. *Cell Oncol.* 2006; 28:127–139. [PubMed: 16988468]
2. Massoud TF, Gambhir SS. Molecular imaging in living subjects: seeing fundamental biological processes in a new light. *Genes Dev.* 2003; 17:545–580. [PubMed: 12629038]
3. Dunn KW, Sutton TA. Functional studies in living animals using multiphoton microscopy. *ILAR J.* 2008; 49:66–77. [PubMed: 18172334]
4. Pawley, JB., editor. *Handbook of Biological Confocal Microscopy*. 3rd. Springer; New York: 2006.

5. Adeghate E, Ponery AS, Ahmed I, Donath T. Comparative morphology and biochemistry of pancreatic tissue fragments transplanted into the anterior eye chamber and subcutaneous regions of the rat. *Eur J Morphol.* 2001; 39:257–268. [PubMed: 12221507]
6. Katoh N, et al. Target-specific innervation by autonomic and sensory nerve fibers in hairy fetal skin transplanted into the anterior eye chamber of adult rat. *Cell Tissue Res.* 1991; 266:259–263. [PubMed: 1722432]
7. Olson L, Seiger A. Beating intraocular hearts: light-controlled rate by autonomic innervation from host iris. *J Neurobiol.* 1976; 7:193–203. [PubMed: 178826]
8. Wu W, Scott DE, Reiter RJ. Transplantation of the mammalian pineal gland: studies of survival, revascularization, reinnervation, and recovery of function. *Exp Neurol.* 1993; 122:88–99. [PubMed: 8101823]
9. Hoffer B, Seiger A, Ljungberg T, Olson L. Electrophysiological and cytological studies of brain homografts in the anterior chamber of the eye: maturation of cerebellar cortex in oculo. *Brain Res.* 1974; 79:165–184. [PubMed: 4424939]
10. Niederkorn, Jy. Immune privilege in the anterior chamber of the eye. *Crit Rev Immunol.* 2002
11. Adeghate E. Pancreatic tissue grafts are reinnervated by neuro-peptidergic and cholinergic nerves within five days of transplantation. *Transpl Immunol.* 2002; 10:73–80. [PubMed: 12182468]
12. Adeghate E. Host-graft circulation and vascular morphology in pancreatic tissue transplants in rats. *Anat Rec.* 1998; 251:448–459. [PubMed: 9713983]
13. Speier S, et al. Noninvasive *in vivo* imaging of pancreatic islet cell biology. *Nat Med.* 2008; 14:574–578. [PubMed: 18327249]
14. Bertera S, et al. Body window-enabled *in vivo* multicolor imaging of transplanted mouse islets expressing an insulin-Timer fusion protein. *BioTechniques.* 2003; 35:718–722. [PubMed: 14579736]
15. Martinic MM, von Herrath MG. Real-time imaging of the pancreas during development of diabetes. *Immunol Rev.* 2008; 221:200–213. [PubMed: 18275484]
16. Zhuravleva ZN, Bragin AG, Vinogradova OS. Organization of the nervous tissue (hippocampus and septum) developing in the anterior eye chamber. I. General characteristic and non-neural elements. *J Hirnforsch.* 1984; 25:313–330. [PubMed: 6470467]
17. Adeghate E, Donath T. Distribution of neuropeptide Y and vasoactive intestinal polypeptide immunoreactive nerves in normal and transplanted pancreatic tissue. *Peptides.* 1990; 11:1087–1092. [PubMed: 2087433]
18. Boutet de Monvel J, Le Calvez S, Ulfendahl M. Image restoration for confocal microscopy: improving the limits of deconvolution, with application to the visualization of the mammalian hearing organ. *Biophys J.* 2001; 80:2455–2470. [PubMed: 11325744]
19. Köhler M, et al. On-line monitoring of apoptosis in insulin-secreting cells. *Diabetes.* 2003; 52:2943–2950. [PubMed: 14633855]
20. Berney T, et al. Endotoxin-mediated delayed islet graft function is associated with increased intra-islet cytokine production and islet cell apoptosis. *Transplantation.* 2001; 71:125–132. [PubMed: 11211177]
21. Nyqvist D, Köhler M, Wahlstedt H, Berggren PO. Donor islet endothelial cells participate in formation of functional vessels within pancreatic islet grafts. *Diabetes.* 2005; 54:2287–2293. [PubMed: 16046293]
22. Bernd AS, Aihara M, Lindsey JD, Weinreb RN. Influence of molecular weight on intracameral dextran movement to the posterior segment of the mouse eye. *Invest Ophthalmol Vis Sci.* 2004; 45:480–484. [PubMed: 14744888]
23. Aynsley-Green A, Biebuyck JF, Alberti KG. Anaesthesia and insulin secretion: the effects of diethyl ether, halothane, pentobarbitone sodium and ketamine hydrochloride on intravenous glucose tolerance and insulin secretion in the rat. *Diabetologia.* 1973; 9:274–281. [PubMed: 4767367]
24. Brown ET, Umino Y, Loi T, Solessio E, Barlow R. Anesthesia can cause sustained hyperglycemia in C57/BL6J mice. *Vis Neurosci.* 2005; 22:615–618. [PubMed: 16332272]

25. Desborough JP, Jones PM, Persaud SJ, Landon MJ, Howell SL. Isoflurane inhibits insulin secretion from isolated rat pancreatic islets of Langerhans. *Br J Anaesth.* 1993; 71:873–876. [PubMed: 8280557]

Figure 1. Imaging setup for noninvasive *in vivo* imaging in the anterior chamber of the eye. Photograph shows an upright microscope with custom-built stage (1) for incorporation of the holding equipment, including metal plate (2), head holder (3), universal joint (4) and heating pad (5).

Figure 2.

Noninvasive *in vivo* imaging in the anterior chamber of the eye. **(a)** The head of the mouse is restrained in the head holder with the eye containing the transplanted pancreatic islets facing upward. Anesthesia is applied via the gas mask. The eye lids of the graft-bearing eye are held back by a polyethylene tubing loop between the tips of a forceps to expose and stabilize the eye. Viscotears is applied as immersion liquid between the objective APO 10 × 0.3 W and the eye. **(b)** Head holder equipped with nose piece for fixation. **(c)** Head holder equipped with gas mask for fixation and simultaneous application of gas anesthesia.

Figure 3. Custom-built stabilizer of the mouse eye for noninvasive *in vivo* imaging. A forceps is attached to a Universal Joint (black) via a metal bar and the tips of the forceps covered with a loop of polyethylene tube. At the front part of the forceps a screw enables adjustment of the loop size. The universal joint is attached to a metal plate (silver), which is covered by a heating pad (white). The head holder was removed for this picture.

Figure 4. Perfusion of the anterior chamber of the mouse eye. Two micropipettes are penetrating the cornea at opposite sides of the eye and allow flow of a fluorescent indicator from the inflow pipette (right) through the anterior chamber into the outflow pipette (left).

Figure 5.

Morphological characterization of a pancreatic islet graft by imaging reflection and GFP. Islets of mice expressing GFP under the insulin promoter (green β cells) were transplanted to mice expressing GFP under the Tie2 promoter (green endothelial cells). **(a)** GFP was excited with 488 nm at 35% laser power and emission measured between 495 and 530 nm. **(b)** Reflection was imaged by exciting with 633 nm at 35% laser power and measuring emission between 632 and 639 nm. **(c)** Overlay of a and b. Objective APO 10 $\times$ 0.3 W. Pinhole: 1 airy unit. Zoom factor: 1.7. Image bit depth: 8 bit. Resolution xyz: 1.38 $\times$ 1.38 $\times$ 5 μ m. Scale bar: 100 μ m.

Figure 6.

Imaging of tissue vascularization. Panels show the same graft as in Figure 5. Blood vessels were visualized by an intravenous injection of a 70 kDa Texas Red labeled dextran. GFP (a) and Texas Red (b) were excited with a two-photon laser at 890 nm at minimal necessary laser power required and emission collected onto nondescanned detectors using a dichroic mirror (RSP 560) and emission filters (BP 525/50 and BP 640/20). (c) Overlay of a and b. Objective APO 10 × 0.3 W. Pinhole: 1 airy unit. Zoom factor: 1.7. Image bit depth: 8 bit. Resolution xyz: $1.38 \times 1.38 \times 5 \mu\text{m}$. Scale bar: 100 μm .

Figure 7.

Imaging of cell death. Panels show the same graft as in Figures 5 and 6. β cell death was induced by intravenous injection of alloxan. Apoptotic and dead cells were visualized by intravenous injection of annexin V-APC. (a) Reflection was imaged by exciting with 543 nm and emission measured between 539 and 547 nm at 35% laser power. (b) GFP was excited at 488 nm and emission measured between 495 and 530 nm at 35% laser power. (c) Allophycocyanin (APC) was excited at 633 nm with collection of emission light between 645 and 680 nm at 75% laser power. (d) Overlay of a,b and c. Objective APO 10 $\times$ 0.3 W. Pinhole: 1 airy unit. Zoom factor: 1.7. Image bit depth: 8 bit. Resolution xyz: 1.38 $\times$ 1.38 $\times$ 10 μ m. Scale bar: 100 μ m.

Figure 8.

Loading of cells within the anterior chamber of the eye with calcium indicators. For loading, the anterior chamber was perfused with Fluo-4 and Fura-Red. Fluo-4 (**a**) and Fura-Red (**b**) were excited with 488 nm at 25% laser power and emission measured for Fluo-4 between 495 and 535 nm and for Fura-Red between 600 and 700 nm. Reflection was imaged by exciting with 543 nm and emission measured between 539 and 547 nm at 15% laser power. (**c**) Overlay of a and b. Objective APO 20 × 0.5 W. Pinhole: 1 airy unit. Zoom factor: 1.7. Image bit depth: 12 bit. Resolution xyz: 0.7 × 0.7 × 3.3 μm. Scale bar: 100 μm.

Table 1

Troubleshooting table.

Step	Problem	Possible reason	Solution
17	Movement of the graft during imaging	Movement of the head because the head holder is not properly fixed	Fix the clamping of the head holder
		Movement of the eye during normal breathing	Adjust the eye stabilizer carefully
		Gaspings of the mouse	Make sure that the airflow of the anesthesia unit and isoflurane levels are set properly
18B(iii)	No fluorescence can be detected in the blood vessels	Tail vein injection failed	Repeat tail vein injection
		Excitation and emission settings are wrong	Adjust excitation and emission settings
		Blood circulation to the eye is disrupted because of false setting of eye stabilizer	Adjust the position of the eye stabilizer
18D(ix)	Perfusion does not work	Air bubbles block the pipettes, capillary holder or tubing	Make sure that there are no air bubbles in the tubing, capillary holders or capillaries prior to the experiment
		Dirt blocks the pipette tips	Be sure to clean the pipettes after beveling, right before the experiment