



Assessment of the In Vivo Response to Nanobody-Targeted PDT Through Intravital Microscopy

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Abstract

Methods that allow real-time, longitudinal, intravital detection of the fluorescence distribution and the cellular and vascular responses within tumor and normal tissue are important tools to obtain valuable information when investigating new photosensitizers and photodynamic therapy (PDT) responses. Intravital confocal microscopy using the dorsal skinfold chamber model gives the opportunity to visualize and determine the distribution of photosensitizers within tumor and normal tissue. Next to that, it also allows the visualization of the effect of treatment with respect to changes in vascular diameter and blood flow, vascular leakage, and tissue necrosis, in the first days post-illumination. Here, we describe the preparation of the skinfold chamber model and the intravital microscopy techniques involved, for a strategy we recently introduced, that is, the nanobody-targeted PDT. In this particular approach, photosensitizers are conjugated to nanobodies to target these specifically to cancer cells.

Key words Intravital microscopy, Colocalization, Targeted photodynamic therapy, Vascular response

1 Introduction

The response to photodynamic therapy (PDT) is the result of the formation of highly reactive singlet oxygen that has a short lifetime and therefore a limited diffusion range. Because of this, the tissue and cellular localization of a photosensitizer is an important parameter, as it determines the primary target of PDT. For example, PDT using a photosensitizer that localizes at the mitochondrial membrane will initially lead to mitochondrial damage, whereas a drug that localizes on the cellular membrane will initially lead to membrane damage. After the initial damage is inflicted, a cascade of secondary responses lead to cellular (e.g., apoptosis and/or necrosis), vascular (e.g., constriction, leakage, and/or stasis), and even immunological responses that eventually determine the PDT efficacy.

For any new (targeted) photosensitizer that shows potential in *in vitro* studies, it is crucial to investigate the drug distribution in tumor and normal tissue, in combination with the cellular and vascular responses to the treatment *in vivo*. Over the years this has been done in several ways using different techniques and various animal models [1–5].

Intravital microscopy (IVM) using a chamber model transplanted with a tumor provides real-time, longitudinal images to determine both the spatial distribution of the (targeted) photosensitizer in time after administration and the vascular and cellular responses to treatment. The advantage of IVM is that the dynamic processes of both drug distribution and treatment responses can be studied longitudinally in real time in the same animal. There are different approaches possible to aid colocalization image analysis, such as (a) labeling tumor cells with GFP, which enables the identification of single tumor cells; (b) employing transgenic mice with tissue-specific fluorescent cells, for example endothelial cells; or (c) administration of fluorescent blood marker to highlight the lumen of the blood vessels [6]. A range of observation chamber models, dorsal skin, brain, breast, and abdominal, are available in a variety of species, most often mice and rats, that can be used for this type of research. The choice of species, model, and tumor line will be determined by the characteristics of the photosensitizer under investigation. Although the animal model (mouse/rat, transgenic/normal, type of observation chamber) and tumor line (GFP expressing or not) may be different, the intravital microscopy procedure will be similar.

In this chapter, we describe IVM using a mouse dorsal skinfold chamber model transplanted with an oral squamous cell carcinoma overexpressing the epidermal growth factor receptor (EGFR) [7]. This setting was used to investigate an EGFR-targeted nanobody-photosensitizer conjugate, in a recently introduced approach [8]. This approach makes use of small antibody fragments derived from heavy-chain antibodies present in Camelids [9], and has shown encouraging results in an orthotopic model of oral squamous cell carcinoma, as it induced extensive tumor damage (80–90%), with minimal damage to surrounding normal tissues [10]. Interestingly, the small size of the nanobody leads to rapid distribution and clearance of the unbound fraction, allowing light activation 1 h postinjection of the conjugate. With IVM, the distribution of the nanobody-photosensitizer conjugate is investigated within tumor and normal tissue by recording fluorescence images using different magnifications at various time points after administration. Image analysis will result in a fluorescence kinetics curve for different tissue types. Colocalization analysis results in a correlation value for the photosensitizer fluorescence and the GFP signal of the tumor. PDT responses are investigated in the same model using IVM. For determination of changes in vascular architecture,

transmission images are recorded up to a maximum of 2 days posttreatment using a low magnification. Vascular shutdown or leakage can be investigated at a specific time point post-PDT by administering a fluorescent blood marker that is large enough not to extravagate the tumor blood vessels, and performing fluorescence imaging and image analysis. Vascular shutdown is then shown by lack of fluorescence, and leakage is visualized by fluorescence outside the vasculature. Normal histology can be used at 2 days posttreatment, on harvested tissue, to evaluate tumor and normal tissue viability or necrosis.

2 Materials

2.1 Mice

SPF-free nude mice of 6 weeks or older and preferably above 18 g are used, as younger and/or lighter mice tend to have less skin. Mice should be fed a complete but chlorophyll-free diet as pheophorbide a, a breakdown product of chlorophyll, is fluorescent and will interfere with the detection of the fluorescence of the administered photosensitizer.

2.2 Tumor Cell Line

Tumor can be transplanted in the chamber either by injecting a cell suspension or by implanting a small piece of tissue, e.g., 1 mm³ tumor fragment (*see Note 1*). Choose a cell line that:

1. Expresses the target of interest.
2. Grows well in skin within 1–2 weeks.
3. Can be located in the chamber (e.g., fluorescent labeled).

Here we use an EGFR-overexpressing human oral squamous cell carcinoma labeled with GFP.

2.3 Analgesia and Anesthesia Procedure

A NSAID's painkiller is administered subcutaneously pre- and 24 h post-operation preventively (1 mg/kg rimadyl cattle). General anesthesia using isoflurane/O₂ inhalation is used during operation and IVM imaging. Monitor breathing of the animal throughout the procedure and adjust isoflurane flow when needed.

2.4 Equipment and Solutions for the Skinfold Chamber Operation

1. Tumor cell suspension or material (which should be collected before starting the operation and maintained on ice).
2. Heating mattress set at 37 °C.
3. Chlorhexidine (2%) in alcohol.
4. Eye ointment.
5. Saline (0.9% sterile NaCl solution).
6. Operation microscope (optional).
7. Marker.

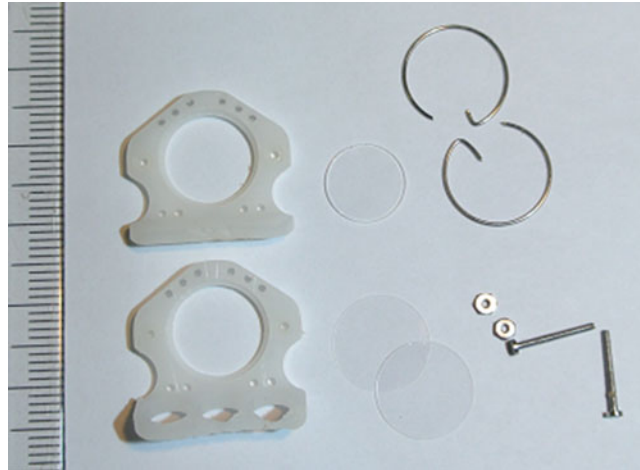


Fig. 1 Dorsal skinfold window chamber parts. Visible are the two frames (white), cover glasses, and filler glass. The retaining rings are thin and do not obscure the view area

8. Skinfold chamber parts as depicted in Fig. 1. Per assembly two window frames (made from PEEK), two pairs of bolts and nuts (aluminum, M1), two retaining rings, two circular cover glasses (nr 1, 0.016 mm thick and 12 mm in diameter), and a 10 mm diameter filler glass. All materials are autoclaved at 105 °C for 90 min.
9. Surgical instruments including silk suture 4/0, 0.5 mL sterile insulin syringe, two 23G needles, scalpel holder + scalpel blade size 15, surgical forceps, surgical microscissors, clamping needle holder (or hemostatic forceps), micro screwdriver, and ear puncher (Fig. 2). All materials are purchased sterile or sterilized by autoclaving for 90 min at 121 °C.

2.5 Equipment and Solutions for Intravital Microscopy

1. Confocal microscope equipped with a heating stage and isoflurane-anesthesia supply unit. The anesthetized animal is placed on the heated stage and, to prevent breathing artifacts while imaging, the chamber of the animal is bolted to a second frame that is then bolted to the heated stage. With this construction the animal can breathe freely while the chamber is fixed. An inverted as well as an upright microscope can be used. Here, we use a Zeiss LSM 510 META confocal microscope.
2. Nanobody-photosensitizer conjugate, in our case a conjugate as in [8, 10].
3. Reference standards.
4. Saline (0.9% sterile NaCl solution).
5. Eye ointment.

5. Fold the dorsal skin, along the folding line, and find the area with clearly visible blood vessels roughly in the middle of the animal. Mark on one side the window part of the frame on the skin above those larger vessels and 2 mm below the folding line and mark the position of the bolts.
6. Use the ear punch to create the holes for the bolts.
7. Excise the dorsal skin within the marked area on one side of the fold, up to the fascia of the opposed skin using the scalpel, surgical microscissors, and forceps.
8. Place the frames with the bolts over the fold and place the screws on the bolts lightly. Pull the skin up 2 mm above the top of the frame and manipulate the tissue, so that the excised fascia area matches the window in the frame. Secure the skin at the top of the frame by putting the 23G needles through the little holes and tighten the screws on the bolts. Replace the 23G needles by sutures and repeat this for the other four sets of little holes in the frame.
9. Place the 10 mm filler glass at the back of the fold in the frame, put a 12 mm cover glass on top, and secure with a retaining ring.
10. Inject 10 μ L tumor cell suspension superficially in the fascia/subcutaneous musculature using a 0.5 mL insulin syringe. Bend the needle to allow easy approach of the tissue. Place the needle between the larger vessels preferably above the middle as superficial as possible. Avoid leaks and repositioning of the needle as spread of cells through the chamber easily occurs. *See Note 1* on how to implant a tumor fragment.
11. Close the window using a 12 mm cover glass and secure it with a retaining ring.
12. Mice are housed solitaire in a climate cabinet set to 32 °C and 70% humidity to avoid hypothermia and dehydration of the skin flap. Use cages and enrichment that allow unobstructed movement and access to food and water.
13. Regularly check the condition of the mice, tissue, and development of the tumor.
14. When the tumor shows signs of growth, IVM experiments can be started.

3.3 IVM

3.3.1 Imaging Photosensitizer Distribution

Using the fluorescence of the nanobody-photosensitizer, the intratumoral distribution of the compound can be monitored over time. Depending on the expected pharmacokinetics of the nanobody-photosensitizer, the time points of interest are chosen (*see Note 4*).

1. Before the animal experiment starts, make sure that you record the reference standard and the dark current on a daily basis (*see Note 5*).

2. Imaging is performed under general inhalation anesthesia, the mouse is fixed onto the heating stage, and eyes receive eye ointment to prevent dehydration.
3. Autofluorescence is recorded before administration of the drug under investigation. Choose the excitation and detection settings fitting with the fluorophores to be imaged. In this case, we used 488 nm excitation and 505–530 nm band-pass detection for the GFP signal of the tumor and 633 nm excitation and long-pass 650 nm detection for the nanobody-photosensitizer conjugate.
4. Image the entire chamber using a low magnification (25×) to identify the tumor and normal tissue and choose the regions to image at a higher magnification (100× and 200×).
5. Determine the time points at which the fluorescence should be recorded depending on the physical and chemical characteristics of the nanobody-photosensitizer (*see Note 4*).
6. Depending on how early is the first measurement time point after administration, the animal is either allowed to wake up after administration or injected on the microscopical stage while under anesthesia.
7. Administer the photosensitizer intravenously using a 0.5 insulin syringe.
8. Record fluorescence images at the specific time points of interest after administration of the drug and, depending on the time slots between these measurements, allow the animal to wake up every time.
9. Record corresponding transmission images to create overlays with the fluorescent images as represented in Fig. 3 to visualize blood vessels (transmitted image), tumor cells (in green), and intra-tumoral distribution of the photosensitizer (in red).

3.3.2 Imaging PDT-Induced Vascular Damage

To image the PDT-induced changes to the vascular architecture of the whole chamber, a similar approach is used. See above for **steps 1–7**.

1. Record fluorescence and transmission before illumination of the photosensitizer.
2. Perform illumination (using the proper laser, wavelength, fluence, and fluence rate), preferably on the microscopic table to minimize loss of time and regions of interest.
3. Record fluorescence and transmission after illumination at various time points after PDT using a low (25×) magnification (*see Note 6*).

To also detect tumor vascular leakage and/or flow, an extra step is introduced.

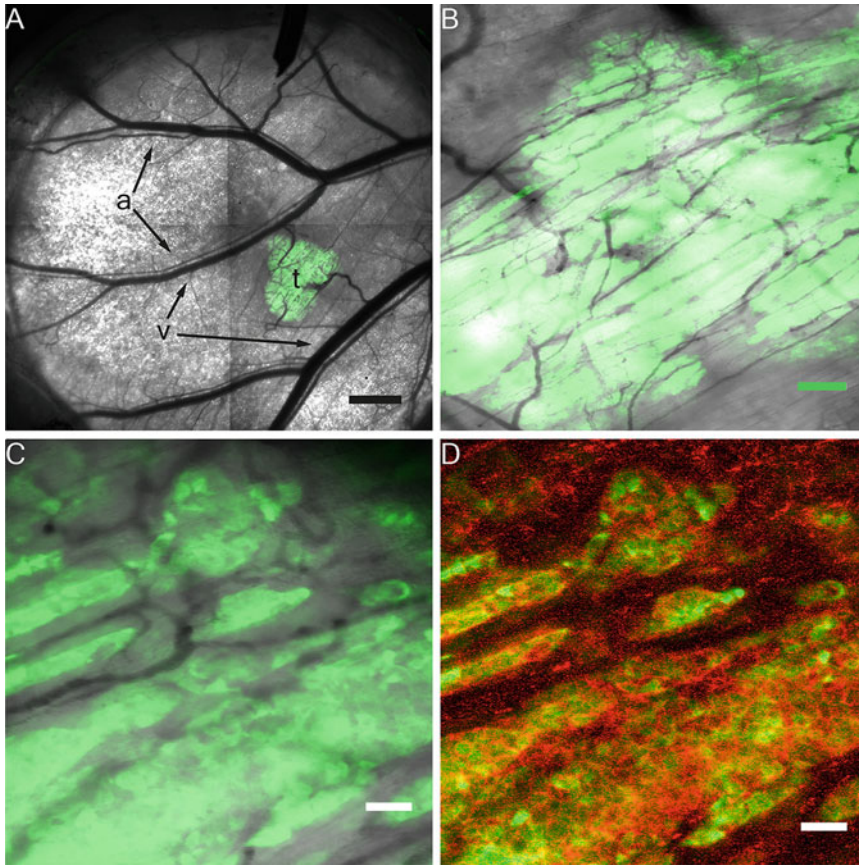


Fig. 3 Examples of fluorescence and transmission images of a skinfold chamber transplanted with a GFP-expressing tumor recorded using different magnifications. **(a)** Transmission and GFP fluorescence image using 25x magnification and composed of 4 stitched images with clearly visible artery (a), venule (v), and tumor (t) tissue. Bar is 1000 μm . **(b)** Transmission and GFP fluorescence image using 100 \times magnification and composed of 4 stitched images. Bar is 200 μm . **(c and d)** Images collected 1 h after administration of a targeted nanobody-photosensitizer (7D12-9G8-PS [7]) using 200 \times magnification showing the transmission and GFP fluorescence **(c)** or GFP and targeted nanobody-photosensitizer fluorescence **(d)**. Bar is 50 μm

4. A fluorescent blood marker is administered intravenously 2 h after illumination (*see Note 7*). 2 MDA tetramethylrhodamine dextran (1 mg/mL, 0.1 mg mouse) remains in the blood-stream for approximately 1 h and will extravagate into the tumor tissue at damaged sites.
5. Rhodamine is detected within 30 min after administration using 543 nm excitation and 560–615 nm band-pass detection.

3.4 Image Analysis

3.4.1 Determining the Fluorescence Kinetics Over Time in Regions of Interest

1. Use ImageJ to analyze the images and Excel to perform all the calculations.
2. Determine the dark current for every imaging setting.
3. Determine the fluorescence intensity of the reference standard for every imaging day.

4. Subtract the reference fluorescence intensity with the corresponding dark current and normalize that to a constant value, preferably close to the mean. Calculate a reference correction factor (=RF) for every imaging day to be used to correct every recorded skinfold chamber image.
5. Draw regions of interest (ROI) around anatomic sites of interest (tumor, normal adipose tissue, blood vessels) in the skinfold chamber images. Save the ROIs accordingly. Determine the fluorescence intensity and save this as raw data.
6. Subtract the raw fluorescence intensity of the ROIs with the corresponding dark current (recorded under the same imaging conditions).
7. Correct for the reference, dividing the fluorescence intensity by the RF.
8. The corrected fluorescence intensity of the individual ROIs can now be used to create the fluorescence kinetic curves.

3.4.2 Colocalization Analysis

As the tumor cells employed express GFP and the photosensitizer conjugated to the nanobodies emits red fluorescence, colocalization analysis can be performed to measure tumor cell-specific accumulation and/or compare different photosensitizers on their performance. The *coloc2* plug-in in ImageJ implements and performs the pixel intensity correlation over space methods of Pearson, Manders, Costes, Li, and more. There are many nuances and pitfalls to colocalization analysis which are described extensively in literature [11–15]. It is highly dependent on the quality of the images taken, which is greatly determined by the quality of the chamber. Avoid fluorescence bleed-through, photobleaching, overexposure, saturation, and difference in intensities between images, as all these determine image quality and result in inferior colocalization analysis.

1. Subtract the dark current from the green and the red images to be analyzed on colocalization.
2. Use the *coloc2* plug-in.
3. Save both the PDF and the txt file containing the data. The file contains the results of all the mentioned colocalization methods.
4. Use the data applicable in your case to report or create figures.

3.4.3 Changes Vascular Architecture in the Chamber

Transmission images recorded with 488 nm light clearly show the vascular architecture while the green light is absorbed by the red blood. Changes in vascular diameter, as a result of constriction, can therefore be visualized and measured/scored. As under transmitted light using 488 nm red blood cells can be seen in the blood flow, vasoconstriction and/or dilatation can be recorded. Until now, it

Table 1**Qualitative score for changes to the vascular architecture in the skinfold chamber treated with PDT**

Score	Description
–	No change in vascular architecture
+	Small changes in vascular lumen size, still flow visible by eye
++	Severe changes in vascular lumen of both the artery and venule, flow visible
+++	Severe changes in vascular lumen of both the artery and venule, no flow visible

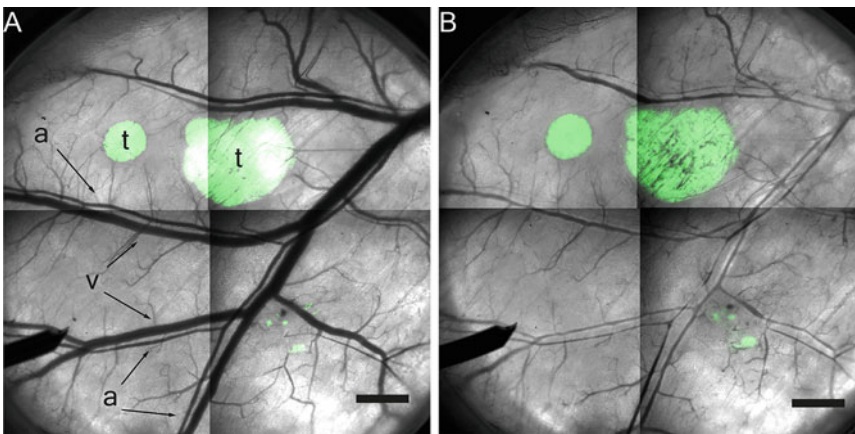


Fig. 4 Example of changes in vascular diameter observed after PDT in the skinfold chamber transplanted with a GFP-expressing tumor (t). (a) Image recorded before PDT showing normal arterial (a) and venule (v) flow. (b) Image showing severe constriction of both venule and artery 2 h after PDT treatment using a targeted nanobody-photosensitizer conjugate [7]. Microscopy observation by eye should be used to determine if this is combined with stasis

has not been possible to use a plug-in that automatically determines the reduced absorption in case of severe constriction. Therefore, we score the severity of the vascular response qualitatively using a scoring table (Table 1, Fig. 4).

3.4.4 Vascular Flow and Leakage in Tumor

Images recorded within 30 min after administration of a blood marker that is large enough not to extravagate from the tumor bloodstream can be used to score for vascular flow and detect leakage. In Fig. 5, vasoconstriction, vascular leakage, and blood flow in the tumor and in the normal vasculature are shown. With respect to flow, it is likely that parts of tumor have different responses; that is, one area in the tumor can show lack of flow, whereas another part shows normal flow. Tumor vascular flow is determined by using the rhodamine fluorescence, GFP fluorescence, and 488 nm transmission image (*see* Fig. 5).

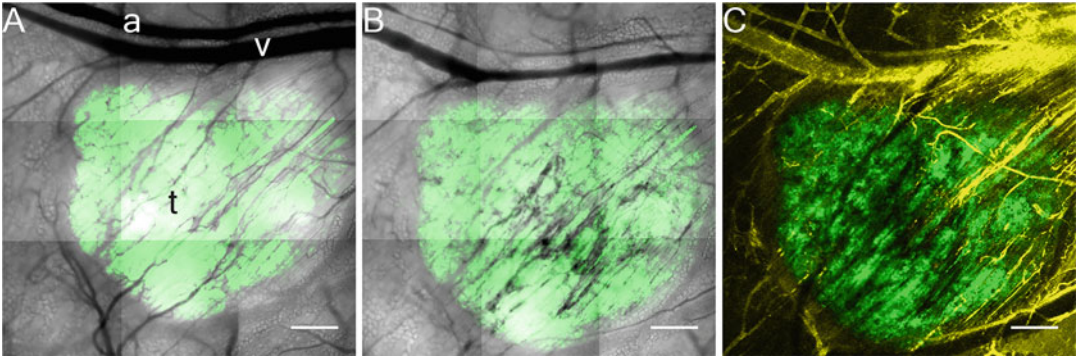


Fig. 5 Example of vascular leakage or stasis observed after PDT in the skinfold chamber transplanted with a GFP-expressing tumor. Rhodamine-dextran 2000 kDa is administered 2 h after PDT and images were recorded within 30 min after administration [7]. **(a)** Transmission and GFP fluorescence of tumor area before PDT. **(b)** Transmission and GFP fluorescence of tumor area showing vasoconstriction of artery and irregular venule diameter and disturbed flow in tumor 2 h after PDT. **C.** GFP and blood marker fluorescence with vascular leakage (l) and areas that show reduced flow (r) or even no flow (no)

1. Train your eye by checking the control animals first: How many vessels are visible in the tumor? Keep this in mind as 100% flow. How much tumor tissue surrounds the vessel? What is the density of the vessels in the tumor?
2. In PDT-treated animals, draw a ROI over the tumor area using the GFP signal.
3. Qualitatively determine if all vessels visible in the transmission image also show rhodamine fluorescence. If so, there is 100% flow.
4. If there are areas that show lack of flow or partial flow, draw a region of interest around this tumor area and determine its proportional size.
5. This data can now be presented in a 100% stacked column graph.
6. With respect to leakage, leakage in tumor or in normal tissue is qualitatively judged and separately scored.

4 Notes

1. To induce the growth of a tumor in a chamber, as an alternative to cell suspension, a tumor fragment can be employed. For that, tumor tissue is harvested from a donor mouse. Procedure: Kill the donor animal and dissect the tumor. Cut pieces of 1 mm³ from the viable part of the tumor and place these in a petri dish with sterile saline on ice. The tumor of the donor mouse should be large enough for sufficient material while

avoiding too large necrotic tumors. To transplant a tumor fragment, create a small pocket in the fascia by using a bent needle and insert the fragment with a microneedle holder. Importantly, tumor material to be transplanted should be collected and stored on ice as short as possible before the operation is started.

2. The skinfold chamber operation should be performed in an operation theatre with an operation microscope. Sterility, experience, proper tools, and instruments are important factors for success. All instruments, materials, and surfaces should be sterilized or cleaned thoroughly with alcohol.
3. When hairy mice are used, shave the back of the animals, apply hair removal gel to remove all hair from the follicles as these are highly autofluorescent, and remove the gel thoroughly.
4. Although it is tempting to keep the mouse on the microscope stage for hours and image the distribution of drugs continuously, prolonged exposure to anesthesia should be avoided as it may influence the distribution of drugs throughout the body. It is better to choose a number of time points at which the fluorescence should be recorded, to be able to create a good pharmacokinetic curve. In this respect, for fast clearing drugs, short intervals, within hours after administration, should be chosen, whereas for longer retaining drugs, intervals up to days after administration can be used.
5. To be able to compare the results of different animals and different experimental days, it is important to image a reference standard and the dark current on a daily basis. The dark current is the background level (offset) of the detector without any light. The dark current of each imaging setting is collected by repeating the image recording while the laser is off. As a reference standard we image a piece of colored glass that shows fluorescence for a specific imaging setting.
6. Vascular responses to PDT can occur very quickly after PDT. Record the change in vascular architecture immediately after treatment using transmission imaging with 488 nm light. Changes in vascular architecture can be monitored up to 2 days post-PDT. After that, the condition of the tissue in the chamber might not be acceptable and overestimation of the vascular effect is a concern.
7. Treatment-induced vascular leakage is a result of damage and/or death of endothelial cells which will take some time to occur. In our case we chose 2 h post-PDT to image vascular leakage.

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