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## Simple and effective administration and visualization of microparticles in the circulatory system of small fishes using kidney injection

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<b>Corresponding Author:</b>	Maxim Timofeyev Institute of Biology at Irkutsk State University Irkutsk, Irkutsk Region RUSSIAN FEDERATION
<b>Corresponding Author's Institution:</b>	Institute of Biology at Irkutsk State University
<b>Corresponding Author E-Mail:</b>	m.a.timofeyev@gmail.com
<b>First Author:</b>	Ekaterina Borvinskaya
<b>Other Authors:</b>	Ekaterina Borvinskaya Anton Gurkov Ekaterina Shchapova Dmitry Karnaukhov Anton Sadovoy Igor Meglinski
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**TITLE:**

**Simple and Effective Administration and Visualization of Microparticles in the Circulatory System of Small Fishes Using Kidney Injection**

**Authors and Affiliations**

Ekaterina Borvinskaya<sup>1,2</sup>, Anton Gurkov<sup>1,3</sup>, Ekaterina Shchapova<sup>1</sup>, Dmitry Karnaukhov<sup>1</sup>, Anton Sadovoy<sup>4</sup>, Igor Meglinski<sup>1,5</sup> and Maxim Timofeyev<sup>1</sup>

<sup>1</sup>Institute of Biology at Irkutsk State University, Irkutsk, Russia

<sup>2</sup>Institute of Biology at Karelian Research Centre of Russian Academy of Sciences, Petrozavodsk, Russia

<sup>3</sup>Baikal Research Centre, Irkutsk, Russia

<sup>4</sup>Institute of Materials Research and Engineering, Agency for Science, Technology, and Research (A\*STAR), Singapore

<sup>5</sup>University of Oulu, Optoelectronics and Measurement Techniques Laboratory, Oulu, Finland

**Corresponding Author**

Maxim Timofeyev (m.a.timofeyev@gmail.com)

Tel: (3952)-243-077

**Email Addresses of Co-authors**

Ekaterina Borvinskaya (borvinska@gmail.com)

Anton Gurkov (a.n.gurkov@gmail.com)

Ekaterina Shchapova (shchapova.katerina@gmail.com)

Dmitry Karnaukhov (karnauhovdmitrii@gmail.com)

Anton Sadovoy (sadovoyav@imre.a-star.edu.sg)

Igor Meglinski (igor.meglinski@oulu.fi)

**Keywords**

*Danio rerio*, microparticles, injection, systemic administration, optical sensor, microencapsulated biomarkers, layer-by-layer assembly, *in vivo* diagnostics, remote physiological measurements

**Summary**

This article demonstrates the principles of a quick, minimally invasive injection of fluorescent microparticles into the circulatory system of small fishes and the *in vivo* visualization of the microparticles in fish blood.

**Abstract**

The systemic administration of micro-size particles into a living organism can be applied for vasculature visualization, drug and vaccine delivery, implantation of transgenic cells and tiny optical sensors. However, intravenous microinjections into small animals, which are mostly used in biological and veterinary laboratories, are very difficult and require trained personnel. Herein, we demonstrate a robust and efficient method for the introduction of microparticles into the circulatory system of adult zebrafish (*Danio rerio*) by injection into the fish kidney. To visualize the introduced microparticles in the vasculature, we propose a simple intravital imaging

technique in fish gills. *In vivo* monitoring of the zebrafish blood pH was accomplished using an injected microencapsulated fluorescent probe, SNARF-1, to demonstrate one of the possible applications of the described technique. This article provides a detailed description of the encapsulation of pH-sensitive dye and demonstrates the principles of the quick injection and visualization of the obtained microcapsules for *in vivo* recording of the fluorescent signal. The proposed method of injection is characterized by a low mortality rate (0-20%) and high efficiency (70-90% success), and it is easy to institute using commonly available equipment. All described procedures can be performed on other small fish species, such as guppies and medaka.

## Introduction

The administration of micro-size particles into an animal organism is an important task in such areas as drug and vaccine delivery<sup>1</sup>, vasculature visualization<sup>2</sup>, transgenic cell implantation<sup>3</sup>, and tiny optical sensor implantation<sup>4,5</sup>. However, the implantation procedure for microscale particles into the vascular system of small laboratory animals is difficult, especially for delicate aquatic organisms. For popular research specimens like zebrafish, it is advised that these procedures be clarified using video protocols.

Intracardiac and capillary microinjections require trained personnel and unique microsurgery facilities for the delivery of microobjects into zebrafish blood. Previously, a retro-orbital manual injection<sup>3</sup>, was suggested as an easy and effective method for the administration of whole cells. However, in our experience, because of the small area of the eye capillary network, it takes much practice to achieve the desired outcome from this technique.

Herein, we describe a method for robust and efficient microparticle implantation into the circulatory system by manual injection directly into the kidney tissue of adult zebrafish, which is rich in capillaries and renal vessels. This technique is based on the video protocol for cell transplantation into the zebrafish kidney<sup>6</sup>, but the traumatic and time-consuming microsurgical steps were eliminated. The proposed method is characterized by low mortality (0-20%) and high efficiency (70-90% success), and it is easy to institute using commonly available equipment.

An important part of the proposed protocol is the visualization of the implanted microparticles (if they are fluorescent or colorized) in the gill capillaries, which allows for the verification of the injection quality, a rough relative assessment of the number of injected particles, and the detection of the spectral signal for physiological measurements directly from the circulating blood. As an example of the possible applications of the described technique, we demonstrate the protocol for *in vivo* measurements of zebrafish blood pH using a microencapsulated fluorescent probe, SNARF-1, originally suggested in Borvinskaya *et al.* 2017<sup>5</sup>.

## Protocol

All experimental procedures were conducted in accordance with the EU Directive 2010/63/EU for animal experiments and have been approved by the Animal Subjects Research Committee of Institute of Biology at Irkutsk State University.



## 1. Fabrication of Microcapsules

Note: Microcapsules carrying a fluorescent dye are prepared using a layer-by-layer assembly of oppositely charged polyelectrolytes<sup>7,8</sup>. All procedures were performed at room temperature.

1.1 To synthesize porous  $\text{CaCO}_3$  microcores enclosing the fluorescent dye, mix 2 mL of the SNARF-1-dextran solution (most polymer-bound fluorescent dye such as FITC-BSA can be used) at a concentration of  $\sim 2$  mg/mL with 0.6 mL each of 1 mol/L solutions of  $\text{CaCl}_2$  and  $\text{Na}_2\text{CO}_3$  under fast stirring.

Note: Pay attention to the different sensitivities of fluorescent dyes to photobleaching; if a light-sensitive fluorescent probe (like SNARF-1) is used, the manipulation and storage of the microparticles must be performed with as little light as possible.

1.2 After 5-10 s of agitation, transfer the suspension to 2 mL microcentrifuge tubes and centrifuge for 15 s at 10,000-12,000 g to pellet  $\text{CaCO}_3$  microcores.

1.3 Discard the supernatant, wash the cores with  $\sim 2$  mL of deionized water, and resuspend the pellet by shaking.

1.4 Repeat the centrifugation-washing procedure three times in total. After the last centrifugation, discard the supernatant.

1.5 Incubate the microcores for 1 min in an ultrasonic bath to reduce their aggregation.

CAUTION: Do not forget to protect ears with headphones.

1.6 To deposit the first polymeric layer on the templates, resuspend the cores in  $\sim 2$  mL of a 4 mg/mL solution of poly(allylamine hydrochloride) (PAH) in 1 mol/L NaCl.

1.6.1 Keep the microcores in the solution for  $\sim 5$  min with constant shaking.

1.6.2 After 15 s of centrifugation, discard the supernatant with the unbound PAH. Wash the covered microcores with deionized water at least 3 times through multiple centrifugation and washing steps. After the last centrifugation, discard the supernatant.

1.6.3. Incubate the microcores for 1 min in an ultrasonic bath to reduce their aggregation.

Note: If the applied fluorescent dye is cationic, start from poly(sodium 4-styrenesulfonate) (PSS) in 1 mol/L NaCl (see step 1.7).

1.7 Repeat step 1.6 with  $\sim 2$  mL of a 4 mg/mL solution of PSS (also containing 1 mol/L NaCl) to deposit the second polymeric layer on the templates.

1.8 Repeat steps 1.6 and 1.7 six times to deposit 12 polymeric layers.

Note: It is not recommended to take a long break (~12 h or more) in the procedure until ~3-5 layers have been deposited because  $\text{CaCO}_3$  microcores without coverage may tend to recrystallize. Note that PSS coverage causes a higher aggregation of the microcores, and the long pause is advisable only when PAH or poly-L-lysine grafted with polyethylene glycol (PLL-g-PEG) is the outmost layer.

1.9 Incubate the covered microcores in 2 mg/mL PLL-g-PEG (~1 mL per microtube) for at least 2 h.

1.9.1 Wash the microcores with water via sequential centrifugation and resuspension steps. After the last centrifugation, discard the supernatant.

1.10 To obtain hollow microcapsules, dissolve the  $\text{CaCO}_3$  templates by adding 2 mL of 0.1 mol/L ethylenediaminetetraacetic acid (EDTA) solution (adjusted to pH 7.1 with NaOH) to the covered microcores.

1.10.1 After ~5 min of incubation, centrifuge the microcapsules for 45 s and discard the supernatant with the EDTA.

1.10.2 Repeat steps 1.10-1.10.1 twice.

1.11 Wash the microcapsules with 0.9% NaCl three times through multiple centrifugation steps within 45 s followed by washing steps. After the last centrifugation step, discard the supernatant.

Note: The final microcapsule solution for injection must be kept sterile (for example by adding ampicillin, 0.1 mg/mL), and the media should be biocompatible with the object of investigation (isotonic media with neutral pH).

1.12 Estimate the concentration of the prepared microcapsules in a hemocytometer under a fluorescence microscope. Take a series of pictures of the microcapsules, measure the diameter of about a hundred microcapsules using ImageJ<sup>9</sup> or equivalent software, and investigate the size distribution using a histogram.

1.13 Store the obtained encapsulated probe in the dark.

Note: After several washings in sterile 0.9% NaCl, the microcapsules can be stored for months at 4 °C. Complete drying of the microcapsules during storage is not recommended.

## **2. Preparation of Optical Setup and Calibration of Microencapsulated SNARF-1**

Note: Rough pH measurements with microencapsulated SNARF-1 can be made using images in

two channels of a fluorescent microscope<sup>7</sup>, but in this protocol a one-channel fluorescent microscope connected to a fiber spectrometer was applied.

2.1 Place the required set of fluorescence filters to the fluorescent microscope according to the characteristics of the applied fluorescent dye and turn on the fluorescent lamp.

2.1.1 Pull out the lever to the eyepieces.

CAUTION: Excess light can damage the spectrometer matrix. Thus, make sure that the lever is in the "eyepiece" mode when the spectrometer is not used.

2.1.2 Connect one end of the optical fiber to the spectrometer and the other end to a collimator. Using adapters, place the collimator in the focus of the camera tube or other available port of the fluorescent microscope.

2.1.3 Turn on the spectrometer. Run the spectrometer control program and prepare the spectrometer for measurements.

2.2 For calibration of the microcapsule batch, place ~5  $\mu\text{L}$  of the microcapsule suspension (~10 000 microcapsules per  $\mu\text{L}$  in deionized water) on a microscope slide, and dry the drop in a dark place (for example, in a thermostat at 35 °C).

2.2.1 To calibrate the spectral characteristics of the microencapsulated SNARF-1, use a series of buffers with different pH values in the range ~6-9. Drop ~10  $\mu\text{L}$  of a buffer onto the dried microcapsules with SNARF-1-dextran and cover it with a coverslip.

2.2.2 Place the glass slide on the microscope stage. Locate the microcapsules using a  $\times 40$  objective.

2.2.3 Turn the microscope lever to the camera port. Register their fluorescence with the spectrometer. Turn the lever back to the eyepieces.

Note: Make sure the spectral signal is far beyond the background level, and ensure that the microcapsules in the field of view are not in a bubble (by switching to a lower magnification if necessary). Avoid prolonged illumination of the same microcapsules. SNARF-1 is sensitive to photobleaching.

2.2.4 Repeat step 2.2.3 for different microcapsules 10-15 times.

2.3 Calculate the fluorescence peak ratios (for example, using R or Scilab) for all registered spectra and determine the regression line between the median ratio (for each buffer) and medium pH using the following formula:

$$pH = a \log \left( \frac{I_{605}/I_{660} - 0.15}{1.1 - I_{605}/I_{660}} \right) + b$$

Note: SNARF-1 has a spectrum with two peaks corresponding to the emission of the protonated and deprotonated dye, and the ratio between the peaks is responsive to the pH of the medium. In this study, the ratio between the fluorescence intensity for 605 and 660 nm is used. These wavelengths are chosen depending on the filter set used.  $a$  and  $b$  are coefficients to be determined by non-linear regression (for example, using R). Values 0.15 and 1.1 are, respectively, the minimum and maximum values of  $I_{605}/I_{660}$  observed during the calibration.

2.4 Collect about 10  $\mu$ L of fish blood from approximately 5 adult animals. Place fish into a Petri dish with 1  $\mu$ L/mL water suspension of clove oil for anesthesia and wait until the animal turns on its side and stops responding to pinches of the fin (usually ~2-3 min). Transfer the fish on a glass slide. Cut off the fish tail with a lancet and collect approximately 2  $\mu$ L of fish blood from the tail vein.

Note: To prevent blood clotting, treat the incision with heparin (5000 U/mL) and use heparinized glass capillaries and microcentrifuge tubes to collect the blood.

2.4.1 Drip about 10  $\mu$ L of blood with a pipette onto the tip of the microelectrode and determine the pH using a pH-meter.

2.4.2 Drop the blood onto a slide with dried microcapsules and register the ratio of the fluorescence intensity as described for the calibration buffers (steps 2.2 - 2.3).

2.5 Adjust the linear coefficient of the calibration curve to make the curve match the measurements in the fish blood (for more details see Borvinskaya *et al.* 2017<sup>5</sup>).

### 3. Preparation for Injection

3.1 Release the steel needle from the tip of the insulin pen (or syringe) by removing the plastic with a sharp lancet.

Note: Any thin needle ( $\varnothing$ 0.33 mm or less) or glass capillary (usually  $\varnothing$ 1 mm) can be prepared for microinjection<sup>10,11</sup>.

3.2 Insert the needle halfway into the glass microcapillary; quickly and gently solder it using a gas torch.

3.3 Connect the glass microcapillary to the microinjector and flush it with sterile water three times. Ensure that the liquid flows through the needle.

3.4 Fill the system with distilled water.

Note: Make sure there are no bubbles in the system.

#### 4. Injection

4.1 Resuspend the prepared suspension of microcapsules (in sterile 0.9% NaCl, or any other media used for the injections, with a concentration of 0.5 to 6 million microcapsules per microliter) using the ultrasonic bath for 1 min.

Note: Since the microcapsules tend to precipitate, during the following injection, shake the vial with the microcapsules mechanically (using a rotor) or manually every few minutes to resuspend them and prevent their aggregation.

4.2 Place the fish into a Petri dish with anesthetic (0.1 mL/L of clove oil suspended in water) for ~2-3 min. Wait until the fish turns on its side and stops responding to a light pinch of the fin.

4.3 Using a spoon, transfer the fish out of the anesthetic and gently place it on a damp sponge in a lateral position with the head towards the left (for right-handed person) or towards the right (for left-handed person).

4.4 Just before the injection, suck 1-2 mm of air into the glass capillary connected with microinjector. Then, load it with approximately 2  $\mu$ L of the dispersed microcapsules.

Note: Before the injection, the microcapsule solution must be adjusted to the temperature at which the fish are kept.

4.5 Gently stabilize the body of the fish on the sponge with non-dominant hand.

4.5.1 Find the lateral line of the fish. Mentally select a segment that extends from the operculum to the end of the abdominal cavity. Find the middle of this segment. Put the needle 1 mm lower in the ventral direction.

4.5.2 With a scraping movement, gently move the fish scales aside, and make a puncture. Insert the needle into the body at an angle of 45° to the table surface.

4.5.3 Push the needle toward the spine until it carefully rests against it.

4.5.4 Release approximately 1  $\mu$ L of the microcapsules' suspension into the kidney, and slowly withdraw the needle.

Note: To find the proper puncture site more easily, it is useful to practice finding the trunk kidney by transilluminating the fishes using a bottom light, as shown in **Figures 2A and 2B**.

4.6 Rinse the fish from head to tail with a stream of water to remove any spilled microcapsules at the injection site.

## 5. *In Vivo* Visualization

5.1 Use the dissection scissors to remove the gill cover from the fish head and denude the fish gills. Rinse the gills with water.

5.2 Using a spoon, transfer the fish to a microscope slide, and place it on the stage of the fluorescent microscope.

Note: Make sure that the gills of the fish do not dry out during successive procedures. To avoid this, periodically moisten them with water using a Pasteur pipette (approximately every 1-2 min).

5.3 Darken the room and using low magnification (10X objective) inspect the gills to find the fluorescent microcapsules.

Note: When the procedure is used for introduction of some fluorescent particles into fish circulatory system, it is recommended to inspect gills of several individuals for unexpected fluorescent particles before injections. Gills of wild-type zebrafish do not have autofluorescence, but in some cases sporadic fluorescent particles (like food pieces or unicellular symbionts) may be present on gills. If necessary, such particles can be recognized based on their specific shape (for example, food pieces have irregular shape, unlike spherical microcapsules) or fluorescence spectrum (*i.e.*, color).

5.3.1 Switch the lens to a higher magnitude (×40 objective), and position a microcapsule or a group of microcapsules in the center of the field of view.

5.3.2 Turn the lever to the port with a connected spectrometer. Record the spectral signal.

5.3.3 Turn the lever back to the eyepiece.

5.3.4 Repeat the measurements for different microcapsules several times.

5.4 Transfer the fish to the aquarium with proper aeration for recovery.

Note: With minimal practice, it is possible to perform the injection and signal recording at an approximate rate of 2-3 min per fish. The measurement can be repeated for one individual several times with the use of repeated low, harmless doses of anesthesia or another method of fixation. For long-term observation, use a system with continuous anesthesia<sup>12</sup>.

## Representative Results

The obtained results come from one of the three main categories of the presented protocol: the formation of fluorescent microparticles by encapsulation of a fluorescent dye (**Figure 1**), the kidney injection of microcapsules with further visualization in gill capillaries (**Figures 2 and 3**) and,

finally, the *in vivo* spectral recording of SNARF-1 fluorescence to monitor blood pH levels (**Figure 4**).

The layer-by-layer approach using coating of the template CaCO<sub>3</sub> cores enclosing the dye by multiple layers of oppositely charged polymers (PAH and PSS) and an outermost layer of a biocompatible polymer (PLL-g-PEG) is a simple and cost-effective method allowing to encapsulate different fluorescent probes such as SNARF-1-dextran, FITC-BSA or others (**Figure 1A**). As a result, hollow microcapsules of micrometric size with stable semi-permeable elastic shells and loaded with the fluorescent dye are obtained (**Figure 1B**). The microcapsules fabricated by this technique are typically non-uniform, with a normal distribution of particle size and characteristic median size in each batch (**Figure 1C**).

[Place **Figure 1** here]

Robust and efficient microparticle implantation into the circulatory system of small fishes can be performed by manual injection directly into the fish kidney. The puncture in the proper site of the fish body (trunk kidney) is crucial for rapid delivery of microparticles by manual injection. The fish kidney is a hematopoietic organ and contains pigmented melanophores, and thus, it is well colored. Because it is nestled between transparent chambers of the swim bladder, it is easy to identify the organ in the intact animal with weak pigmentation or by transillumination of small fishes using a bottom light source, as shown in **Figures 2A and 2B**.

[Place **Figure 2** here]

To check the success of the injection procedure, a rapid visual inspection of the gills at low magnification (10-20X objective) should be made after cutting the fish gill cover (**Figure 3A**). Despite most of the microcapsules remaining at the injection site or spilling into the body cavity (**Figure 3B**), if the injection is performed correctly, it is possible to observe the fluorescent microcapsules freely floating in the gill capillaries of the denuded fish gills immediately after the injection (**Figure 3C**). If no fluorescent particles are detected in the gills, it is possible to repeat the injection in the same puncture. Successful delivery to the bloodstream should be obtained in approximately 70-90% of the total injected fish.

[Place **Figure 3** here]

During the injection directly into the fish trunk kidney, extensive bruising normally forms beneath the puncture site, indicating damage to the parenchyma capillaries or the renal vessels. There is no blood leakage out of the body because the puncture appears to quickly contract. Despite internal bleeding, individuals can still recover with approximately 80-90% surviving through the procedure (**Table 1**). It is also known that fish species can regenerate nephrons *de novo* after injury<sup>13,14</sup>. If more than 20% of fish are dying, one must ensure that the anesthetization is performed properly.

[Place **Table 1** here]

If the concentration of the injected microparticles is insufficient, too few particles may be available to be rapidly visualized in fish gills. At the same time, a suspension with a too high concentration can clog the needle. In case of layer-by-layer assembled microcapsules with median diameter  $\sim 2\text{-}5\text{ }\mu\text{m}$ , a concentration of approximately  $4 \times 10^5\text{-}6 \times 10^6$  microcapsules per  $\mu\text{L}$  is optimal for effortless detection in fish gills after injection into fish kidney (**Table 2**).

[Place **Table 2** here]

The proposed method of microparticles' implantation into fish circulatory system can be applied for *in vivo* monitoring of zebrafish blood pH by microencapsulated fluorescent probe, SNARF-1 (**Figure 4**). SNARF-1 has a spectrum with two peaks corresponding to the emission of the protonated and deprotonated dye (**Figure 4A**), thus the calibration curves of the ratio between the peaks at different pH of the media can be plotted (**Figure 4B**). The components of the blood influence the readout of the encapsulated SNARF-1 (**Figure 4B**), which can be evaluated experimentally by simultaneous measurement of blood pH by microcapsules and a pH-meter with a glass microelectrode. A putative calibration curve for microcapsules in zebrafish blood should be plotted by shifting the buffers' curve by the coefficient equal to the pH difference measured experimentally (see Borvinskaya *et al.* 2017<sup>5</sup> for details).

Blood pH in zebrafish gill capillaries remains stable during at least several hours after the injection of microcapsules (**Figure 4C**). At the same time, a short hypercapnic exposure leads to a statistically significant decrease of blood pH, which demonstrates applicability of the method for *in vivo* research on small fishes.

[Place **Figure 4** here]

## Figure and Table Legends

**Figure 1: Layer-by-layer synthesis and characterization of hollow polyelectrolyte microcapsules loaded with a fluorescent dye. (A)** Scheme of a microcapsules assembly (drawn from a similar scheme published in Gurkov *et al.* 2016<sup>4</sup>). **(B)** Picture of hollow microcapsules loaded with fluorescent dye FITC-BSA. **(C)** Size characterization of the batch of microcapsules from **Figure 1B**.

**Figure 2: Localization of the proper site for kidney injection. (A)** Trans-illumination of the zebrafish demonstrates the localization of the trunk kidney (arrow). **(B)** Scheme illustrates how to identify the proper puncture site. The white dotted line indicates the lateral line of the abdominal segment of the fish. The arrow indicates the site for puncture and the proper direction of injection toward the spine. The swim bladder is designated by the green line. **(C)** Visualization of zebrafish kidney following the removal of organs and the body wall. An arrow points to the central bulge of the trunk kidney. **(D)** A sagittal histological section of *D. rerio* illustrates the general anatomy of the internal organs of the adult fish (H&E stain). **(E)** Micrograph of a fish kidney (dotted) scaled from (D) with an arrow pointing to the lumen of the posterior cardinal vein. Asterisks indicate the swim bladder.



**Figure 3: Kidney injection of microcapsules with further visualization in gill capillaries.** (A) The overall scheme of the microcapsule delivery into the zebrafish bloodstream. (B) Fluorescent image of a zebrafish after the injection of the microcapsules with green FITC-BSA dye (the puncture site is indicated by an arrow). (C) This picture of the gills of fish, scaled from (B), demonstrates the successful delivery of microcapsules by kidney injection (gills of wild-type zebrafish have no autofluorescence).

**Figure 4: Representative example of monitoring of zebrafish blood pH by registration of the fluorescence spectra of microencapsulated dye SNARF-1.** (A) Spectra of the prepared microcapsules loaded with SNARF-1 in sodium phosphate buffers at different pH. (B) Calibration curves of the prepared pH-sensitive microcapsules in sodium phosphate buffers and in extracted zebrafish blood. For all measurements, the mean  $\pm$  standard deviation is depicted. (C) A representative example of the *in vivo* monitoring of zebrafish blood pH by encapsulated fluorescent SNARF-1 dye in zebrafish gill capillaries. In control conditions, blood pH remains stable during 4 hours after injection of microcapsules, while 5 minutes exposure under severe hypercapnia (900-1000 mg/L of dissolved CO<sub>2</sub>) causes acidification of the fish blood. Asterisk indicates statistically significant difference from the parallel control group with  $p < 0.01$  (Mann–Whitney U test).

**Table 1. Safety and efficiency of the delivery of microcapsules into the zebrafish bloodstream.** The success of the procedure is determined by the presence of the fluorescent materials in the fish gill capillaries.

**Table 2. Record of the visual counting of FITC-BSA-containing microcapsules in *D. rerio* gills after injection (1.6  $\mu$ L) into the trunk kidney.**

## Discussion

To demonstrate the injection of microparticles into the zebrafish kidney, we used semi-permeable microcapsules loaded with an indicator dye. Thus, the protocol contains instructions for the fabrication of the microcapsules using the layer-by-layer assembly of oppositely charged polyelectrolytes<sup>7-8, 15-18</sup> (Figure 1A). An advantage of this technology is that it is easy to perform with available laboratory equipment. Depending on the conditions and compounds used, the fabricated microcapsules can range from 0.5 to 100  $\mu$ m with nanometers-thick polyelectrolyte shell<sup>15</sup>. The synthesis parameters described in this manuscript result in elastic microcapsules consisting of 12 layers (in addition to the final biocompatible layer) of polymers approximately 2-6  $\mu$ m in size (Figures 1B and 1C). The most important step determining the size of the microcapsules is the formation of the template microcores. This process involves the spontaneous formation of calcium carbonate crystals, and therefore, the obtained particles are non-uniform. Hence, a characterization of the microcapsule size distribution should be performed for every batch.

The important stage of this protocol is the optimization of the delivery of microparticles into the zebrafish circulatory system without the use of micromanipulation techniques. The

administration of whole cells by retro-orbital injection has been previously described in detail<sup>3</sup>. However, in our experience, it is not easy for novice users to gain the injection efficiency described by the authors because the retro-orbital sinus is very small and located close to the pharynx and gill arches, which are easy to accidentally injure with a needle. Since less than a millimeter accuracy is required for the injections, the injecting properly is a rather difficult task. An equally quick and more efficient alternative (**Table 1**) is to inject directly into the fish kidney parenchyma. During the injection, the needle mechanically damages renal capillaries and blood vessels (*e.g.*, the largest posterior cardinal vein), which allows entrance of microparticles into the circulatory system (**Figure 2E**). Finally, the central bulge of the trunk kidney in adult zebrafish is large enough (up to 2 mm) for a manual injection without microsurgical equipment.

The proper positioning of the injection needle is critical for a successful manual administration. You can practice finding the trunk kidney in intact animals by transilluminating fishes using a bottom light, as shown in **Figures 2A and 2B**. The scheme in **Figure 2B** demonstrates how to properly perform the injection. If the procedure fails to administer microparticles into the blood stream, re-injection can be made at the same puncture within a short time frame with virtually no effect on the survival rate of the individuals.

Injection into the trunk kidney of adult zebrafish (also successfully tested on adult guppy *Poecilia reticulata*) is an effective method of microparticle delivery into the fish bloodstream with an allowable animal mortality; however, it is not perfectly suitable for drug administration due to variations in the injected volume. A weak point of this technique is a significant leakage of the solution out of the kidney into the abdomen because of the rapid administration. Nevertheless, a relative amount of the substance injected into bloodstream can be roughly estimated using visualization in the gill capillaries (**Table 2**). There is no strict limitation of the injection volume, but the administration of more than 1  $\mu\text{L}$  of the suspension appears to be ineffective. The finest glass capillaries can be applied for the microinjection; however, because a large number of microcapsules tends to incite aggregation, we recommend the use of 31-29G ( $\varnothing$  0.33-0.25 mm) steel needles to avoid clogs in the needle lumen.

The success of microcapsule delivery through kidney injection into the bloodstream should be monitored using the rapid inspection for the presence of the fluorescent particles in the gills. Gills are easily accessible organs for *in vivo* observations. The gill filaments are a network of blood capillaries covered by a thin layer of respiratory epithelium, which makes intravital imaging of the blood flow in the gills particularly convenient (a kind of natural optical window). To perform the observation, the cartilage gill cover can be cut to expose the filaments. This procedure is safe for fish, which can live with denuded gills in well aerated water without a decrease in life expectancy. Moreover, the gills of large fish can be examined under a microscope simply by pushing the operculum out of the way<sup>19</sup>. In case of a successful injection, fluorescent microparticles immediately appear in the gills (**Figure 3**). Note that implanted microparticles are considerably dissolved in the circulating blood volume, and a reduced number of particles reach the gill capillaries, thus sufficient concentration of microparticles should be selected for detection in fish gills after injection into fish kidney (**Table 2**).

The proposed procedure for implantation can be applied in a wide range of studies involving different species of small fishes. Despite the technique having been developed and optimized for injection of fluorescent microparticles into circulatory system, it can also be applied for implantation of non-colored micro/nanoparticles or dissolved substances; however, in this case the effectiveness of injection should be verified in some other way. The currently described steps are optimal for such purposes as physiological monitoring using different optical micro- or nanosensors, visualization of vasculature, delivery of vaccines or drugs on some optically visible carriers, and implantation of genetically modified cells.

## Acknowledgments

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## Disclosures

The authors have nothing to disclose.

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Figure 1

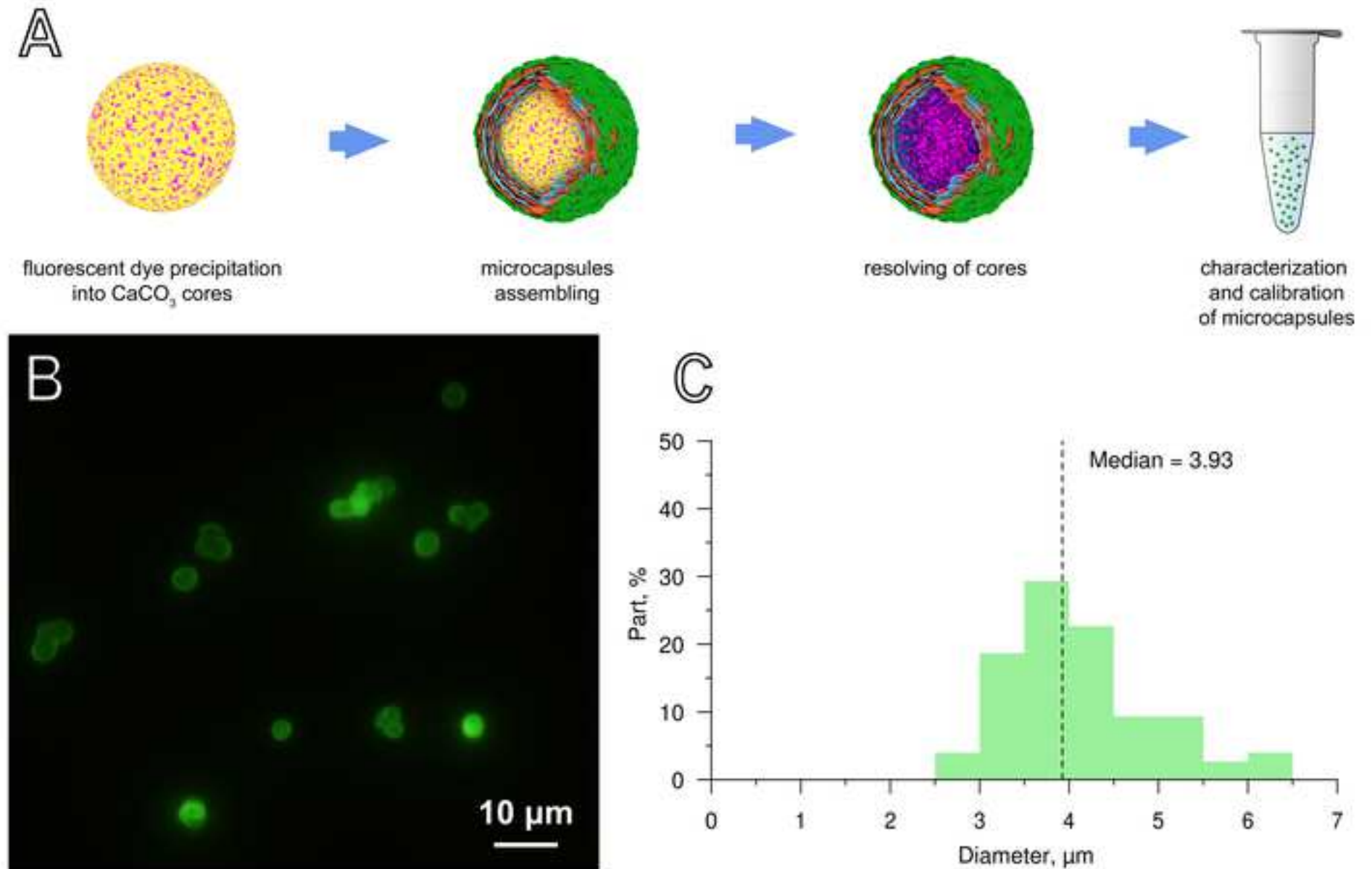
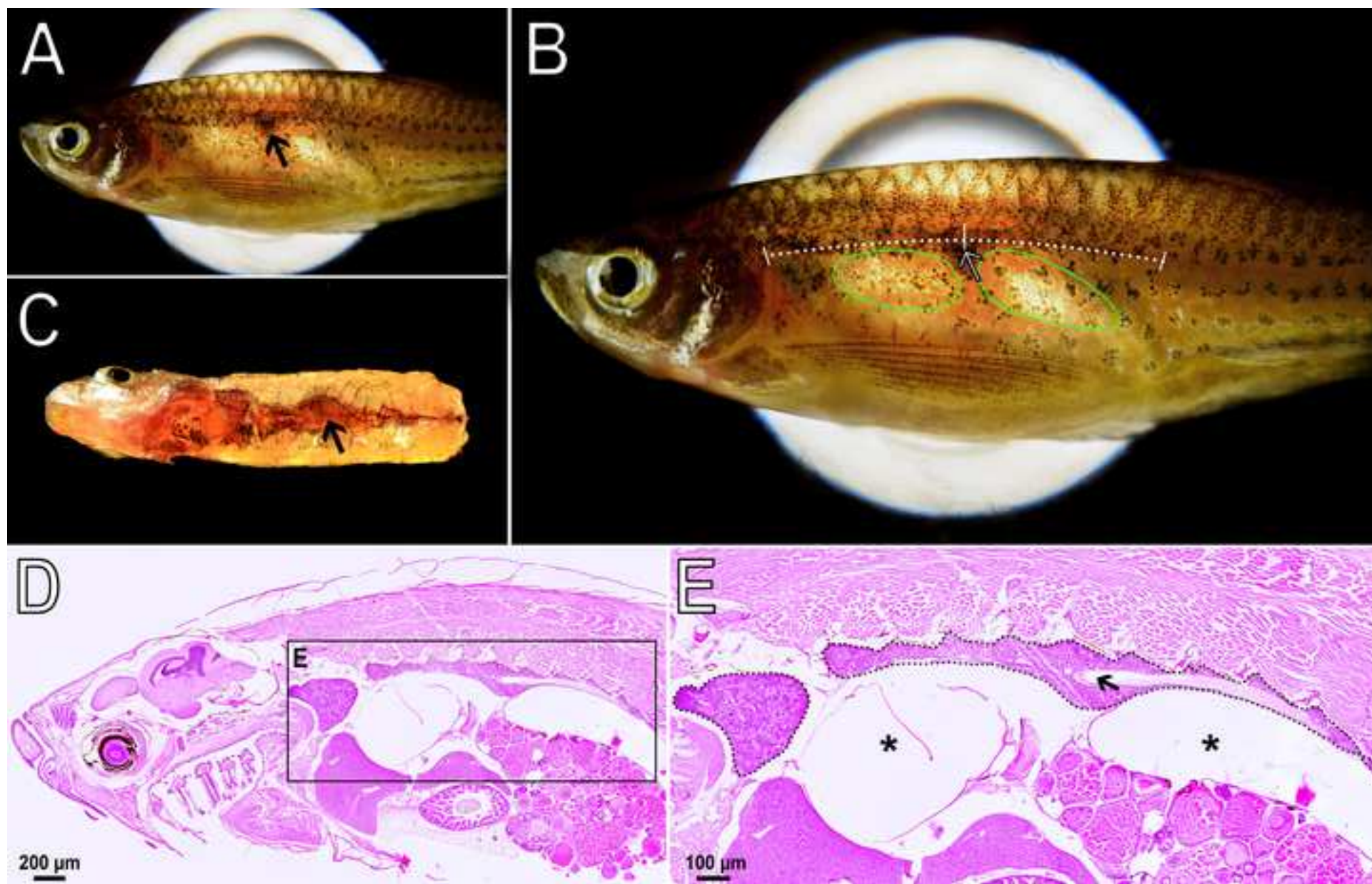


Figure 2

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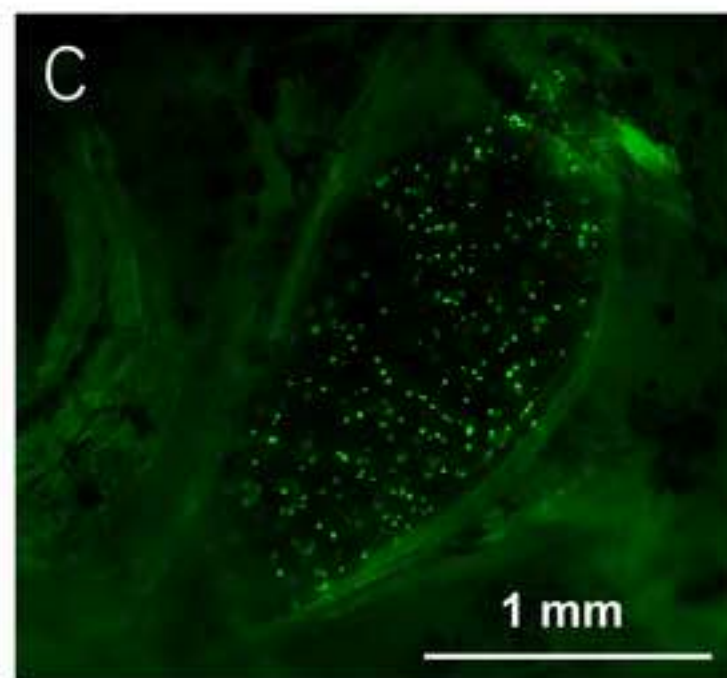
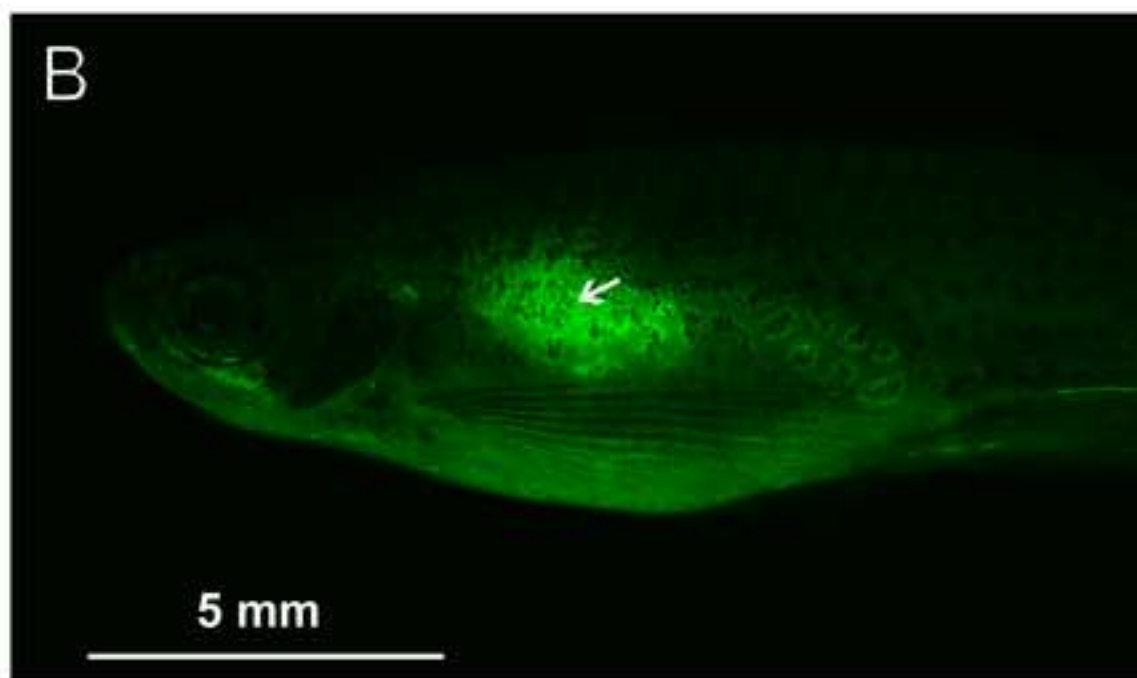
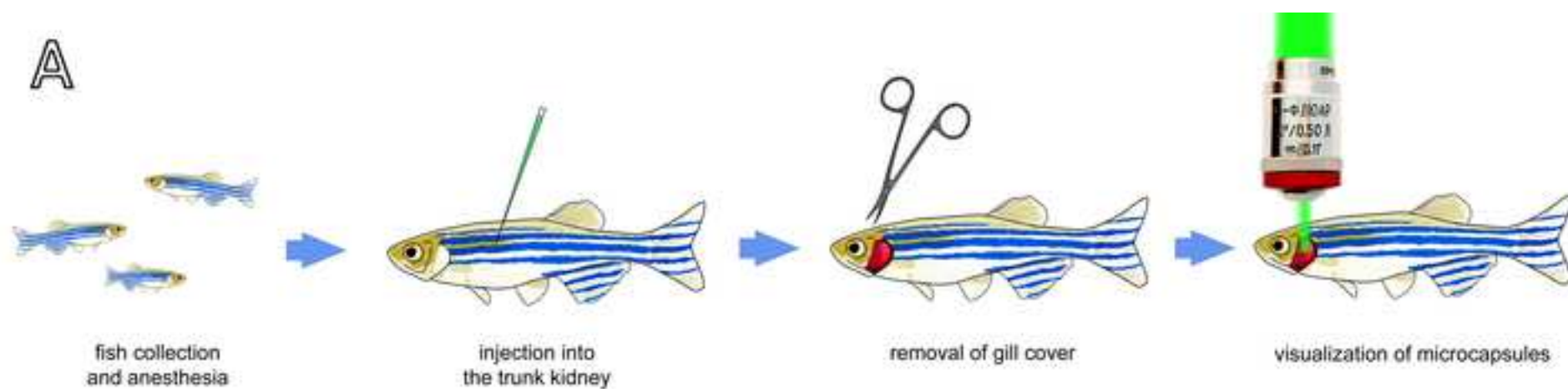
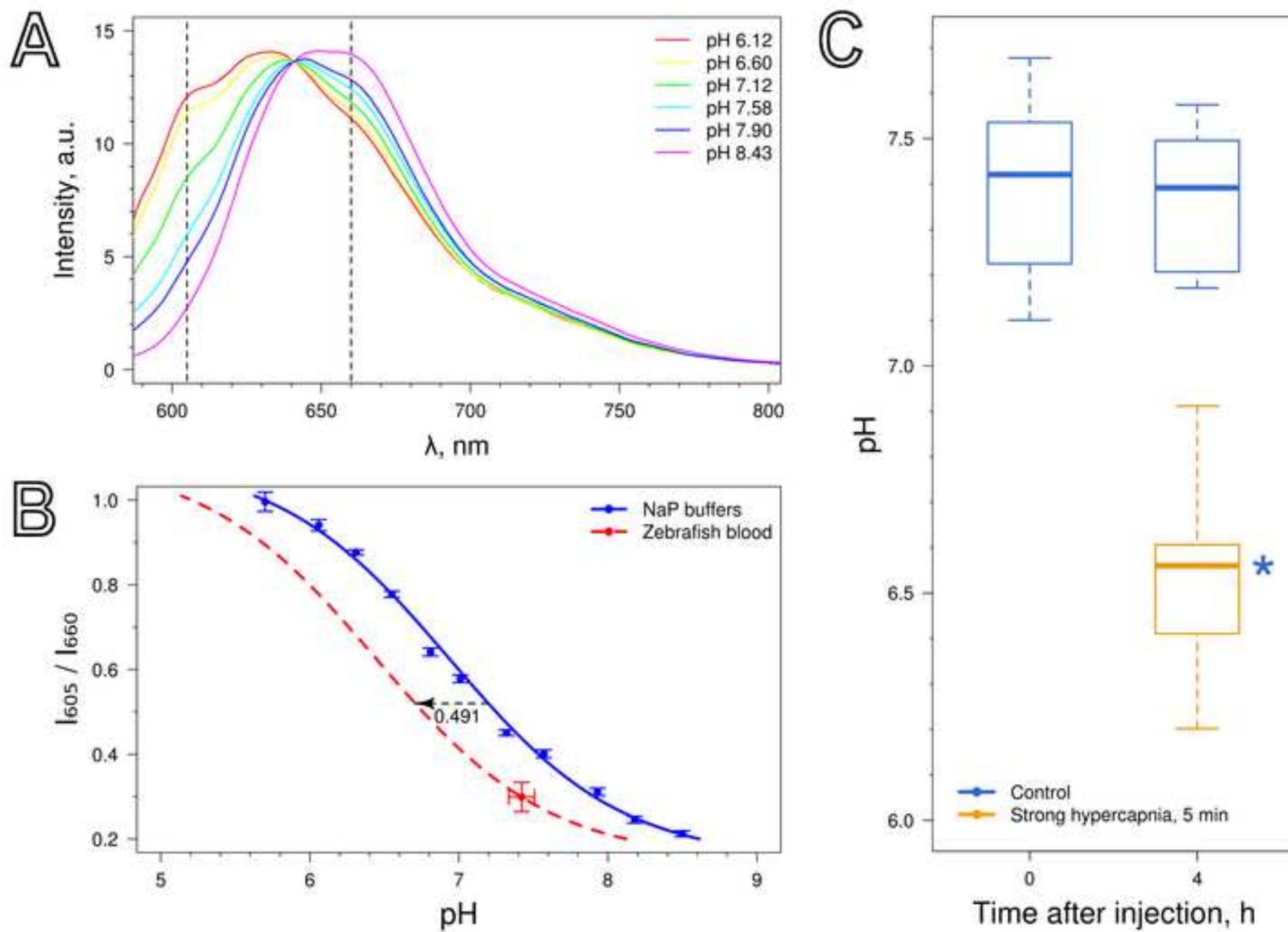


Figure 4

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Encapsulated fluorescent dye	n	Concentration, microcapsules per $\mu\text{L}$	Injection volume, $\mu\text{L}$	Average diameter of microcapsules, $\mu\text{m}$	Successful delivery into the fish bloodstream, %
<b><i>Injection into the trunk kidney</i></b>					
FITC-BSA	36	$4 \times 10^6$	1.6	2.7	94
SNARF-1-dextran	29	$3.6 \times 10^5$	1-2	5.1	72
RITC-dextran	20	$6 \times 10^6$	2	2.0	70
0.9% NaCl	41	-	1-2	-	-
<b><i>Retro-orbital injection</i></b>					
FITC-BSA	9	$1 \times 10^5$	2	4.2	11
RITC-dextran	11	$6 \times 10^6$	1	2.0	9

---

**Mortality after injection, %**

<b>1 h</b>	<b>1 day</b>
8	3
7	12
0	0
5	0
22	0
18	0

---

Concentration, microcapsules per $\mu\text{L}$	Number of microcapsules in a microscope field of view ( $\times 20$ objective) individuals (n = 7 per concentration)				
$4 \times 10^6$	> 100	> 100	0	> 100	$\approx 70$
$4 \times 10^5$	$\approx 10$	$\approx 20$	0	0	$\approx 20$
$4 \times 10^4$	0	3	0	0	0
$4 \times 10^3$	0	0	0	0	0

---

ve) in the gills of different

---

$\approx 50$	$> 100$
$\approx 40$	$\approx 15$
0	4
0	0

---

Name of Material/ Equipment	Company	Catalog Number
SNARF-1-dextran, 70000 MW	Thermo Fisher Scientific	D3304
Albumin-fluorescein isothiocyanate conjugate (FITC-BSA)	SIGMA	A9771
Rhodamine B isothiocyanate-Dextran (RITC-dextran)	SIGMA	R9379
Calcium chloride	SIGMA	C1016
Sodium carbonate	SIGMA	S7795
Poly(allylamine hydrochloride), MW 50000 (PAH)	SIGMA	283215
Poly(sodium 4-styrenesulfonate), MW 70000 (PSS)	SIGMA	243051
Poly-L-lysine [20 kDa] grafted with polyethylene glycol [5 kDa], g = 3.0	SuSoS	PLL(20)-g[3.5]-PEG(5)
Sodium chloride	SIGMA	S8776
Water Purification System	Millipore	SIMSV0000
Magnetic stirrer	Stegler	
Eppendorf Research plus pipette, 1000 $\mu$ L	Eppendorf	
Eppendorf Research plus pipette, 10 $\mu$ L	Eppendorf	
Pipette tips, volume range 200 to 1000 $\mu$ L	F.L. Medical	28093
Pipette tips, volume range 0.1-10 $\mu$ L	Eppendorf	Z640069
Mini-centrifuge Microspin 12, High-speed	BioSan	
Microcentrifuge tubes, 2 mL	Eppendorf	Z666513
Shaker Intelli-mixer RM-1L	ELMY Ltd.	
Ultrasonic cleaner		

Head phones		
Ethylenediaminetetraacetic acid	SIGMA	EDS
Monosodium phosphate	SIGMA	S9638
Disodium phosphate	SIGMA	S9390
Sodium hydroxide	SIGMA	S8045
Thermostat chamber		
Hemocytometer blood cell count chamber		
Fluorescent microscope Mikmed 2	LOMO	
Set of fluorescent filters for SNARF-1 (should be chosen depending on the microscope model; example is provided)	Chroma	79010
Fiber spectrometer QE Pro	Ocean Optics	
Optical fiber QP400-2-VIS NIR, 400 $\mu$ m, 2 m	Ocean Optics	
Collimator F280SMA-A	Thorlabs	
Glass microscope slide	Fisherbrand	12-550-A3
Coverslips, 22 x 22 mm	Pearl	MS-SLIDCV
Glass microcapillaries Intra MARK, 10 $\mu$ L	Blaubrand	BR708709
Clove oil	SIGMA	C8392
Lancet No 11	Apexmed international B.V.	P00588
Heparin, 5000 U/mL	Calbiochem	L6510-BC
Seven 2 Go Pro pH-meter with a microelectrode	Mettler Toledo	

Insulin pen needles Micro-Fine Plus, 0.25 x 5 mm	Becton, Dickinson and Company	
Glass capillaries, 1 x 75 mm	Hirschmann Laborgeräte GmbH	9201075
Gas torch		
Microinjector IM-9B	NARISHIGE	
Petri dishes, 60 mm x 15 mm, polystyrene	SIGMA	P5481
Plastic spoon		
Damp sponge		
Dissection scissors	Thermo Scientific	31212
Pasteur pipette, 3.5 mL	BRAND	Z331767

Comments/Description
Fluorescent probe. Any other appropriate polymer-bound fluorescent dye can be used
Fluorescent probe
Fluorescent probe
CaCO <sub>3</sub> templates formation
CaCO <sub>3</sub> templates formation
Cationic polymer
Anionic polymer
Final polymer to increase the biocompatibility of microcapsules
To dissolve applied polymers
To prepare deionized water
For CaCO <sub>3</sub> templates formation
Dosing solutions
Dosing solutions
Dosing solutions
Dosing solutions
For microcapsule centrifugation-washing procedure
Microcapsule synthesis and storage
To reduce microcapsule aggregation
To reduce microcapsule aggregation



To protect ears from ultrasound
To dissolve the $\text{CaCO}_3$ templates
Preparation of pH buffers
Preparation of pH buffers
To adjust the pH of the EDTA solution and buffers
To dry microcapsules on glass slide
To investigate the size distribution and concentration of the prepared microcapsules
<i>In vivo</i> visualization of microcapsules in fish blood
Visualization of microcapsules with fluorescent probes
Calibration of microcapsules under microscope
To connect spectrometer with microscope port
To connect spectrometer with microscope port
Calibration of microcapsules under microscope
Calibration of microcapsules under microscope
To collect fish blood
Fish anesthesia
To cut the fish tail and release the steel needle from the tip of insulin autoinjector
For treating all surfaces that come in contact with fish blood during fish blood collection
To determine fish blood pH

For injection procedure. Any thin needle ( $\emptyset$  0.33 mm or less) is appropriate

For injection procedure

To solder steel needle to glass capillary

For precise dosing of microcapsules suspension

For manipulations with fish under anesthesia

For manipulations with fish under anesthesia

For manipulations with fish under anesthesia

To remove the gill cover from the fish head

To moisten fish gills



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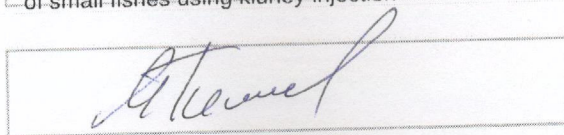
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3. ~ 2:53 (and possibly elsewhere): JoVE's on-screen format calls for subscripts in chemical formulas (e.g.,  $\text{Ca}_2\text{CO}_3$  instead of  $\text{Ca2CO3}$ ).

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MT: It's now corrected.

5. Can you mention somewhere in the manuscript that you have verified this technique with guppies, per your response to Reviewer 2?

MT: It's added to the Discussion.

6. 5.2 note: Around how often should the gills be moistened?

MT: Once per 1-2 min is totally enough. It's added to the Protocol.

7. Figure 3A: 'Microcapsule visualization' or 'visualization of microcapsules' is better usage.

MT: It's now corrected.

Thank you very much again for your help in improvement of the manuscript and protocol.

Very sincerely yours,

Dr. Sci., Prof. Maxim A. Timofeyev

Irkutsk State University

# Simple and effective administration and visualization of microparticles in the circulatory system of small fishes using kidney injection

Ekaterina Borvinskaya <sup>1,2</sup>, Anton Gurkov <sup>1,3</sup>, Ekaterina Shchapova <sup>1</sup>, Dmitry Karnaukhov <sup>1</sup>, Anton Sadovoy <sup>4</sup>, Igor Meglinski <sup>1,5</sup> and Maxim Timofeyev <sup>1</sup>

<sup>1</sup>*Institute of Biology at Irkutsk State University, Irkutsk, Russia.*

<sup>2</sup>*Institute of Biology at Karelian Research Centre of Russian Academy of Sciences, Petrozavodsk, Russia.*

<sup>3</sup>*Baikal Research Centre, Irkutsk, Russia.*

<sup>4</sup>*Institute of Materials Research and Engineering, A\*STAR, Singapore.*

<sup>5</sup>*University of Oulu, Optoelectronics and Measurement Techniques Laboratory, Oulu, Finland.*

*Corresponding Author:*

*Maxim Timofeyev*

*Email Address: m.a.timofeyev@gmail.com*

*Tel: (3952)-243-077*

*Email Addresses of Co-authors:*

*Ekaterina Borvinskaya (borvinska@gmail.com)*

*Anton Gurkov (a.n.gurkov@gmail.com)*

*Ekaterina Shchapova (shchapova.katerina@gmail.com)*

*Dmitry Karnaukhov (karnauhovdmitrii@gmail.com)*

*Anton Sadovoy (sadovoyav@imre.a-star.edu.sg)*

*Igor Meglinski (igor.meglinski@oulu.fi)*

## Short abstract

This article demonstrates the principles of a quick, minimally invasive injection of fluorescent microparticles into the circulatory system of small fishes and the *in vivo* visualization of the microparticles in fish blood.

## Abstract

The systemic administration of micro-size particles into a living organism can be applied for vasculature visualization, drug and vaccine delivery, implantation of transgenic cells and tiny optical sensors. However, intravenous microinjections into small animals, which are mostly used in biological and veterinary laboratories, are very difficult and require trained personnel. Herein, we demonstrate a robust and efficient method for the introduction of microparticles into the circulatory system of adult zebrafish *Danio rerio* by injection into the fish kidney. To visualize the introduced microparticles in the vasculature, we propose a simple intravital imaging technique in fish gills. *In vivo* monitoring of the zebrafish blood pH was accomplished using an injected microencapsulated fluorescent probe, SNARF-1, to demonstrate one of the possible applications of the described technique. This article provides a detailed description of the encapsulation of pH-sensitive



dye and demonstrates the principles of the quick injection and visualization of the obtained microcapsules for *in vivo* recording of the fluorescent signal. The proposed method of injection is characterized by a low mortality rate (0-20%) and high efficiency (70-90% success), and it is easy to institute using commonly available equipment. All described procedures can be performed on other small fish species, such as guppies and medaka.

## **Keywords**

*Danio rerio*; microparticles; injection; systemic administration; optical sensor; microencapsulated biomarkers; layer-by-layer assembly; *in vivo* diagnostics; remote physiological measurements

## **Introduction**

The administration of micro-size particles into an animal organism is an important task in such areas as drug and vaccine delivery<sup>1</sup>, vasculature visualization<sup>2</sup>, transgenic cell implantation<sup>3</sup>, and tiny optical sensor implantation<sup>4, 5</sup>. However, the implantation procedure for microscale particles into the vascular system of small laboratory animals is difficult, especially for delicate aquatic organisms. For popular research specimens like zebrafish, it is advised that these procedures be clarified using video protocols.

Intracardiac and capillary microinjections require trained personnel and unique microsurgery facilities for the delivery of microobjects into zebrafish blood. Previously, a retro-orbital manual injection<sup>3</sup>, was suggested as an easy and effective method for the administration of whole cells. However, in our experience, because of the small area of the eye capillary network, it takes much practice to achieve the desired outcome from this technique.

Herein, we describe a method for robust and efficient microparticle implantation into the circulatory system by manual injection directly into the kidney tissue of adult zebrafish, which is rich in capillaries and renal vessels. This technique is based on the video protocol for cell transplantation into the zebrafish kidney<sup>6</sup>, but the traumatic and time-consuming microsurgical steps were eliminated. The proposed method is characterized by low mortality (0-20%) and high efficiency (70-90% success), and it is easy to institute using commonly available equipment.

An important part of the proposed protocol is the visualization of the implanted microparticles (if they are fluorescent or colored) in the gill capillaries, which allows for the verification of the injection quality, a rough relative assessment of the number of injected particles, and the detection of the spectral signal for physiological measurements directly from the circulating blood. As an example of the possible applications of the described technique, we demonstrate the protocol for *in vivo* measurements of zebrafish blood pH using a microencapsulated fluorescent probe, SNARF-1, originally suggested in<sup>5</sup>.

## **Protocol**

All experimental procedures were conducted in accordance with the EU Directive 2010/63/EU for animal experiments and have been approved by the Animal Subjects Research Committee of Institute of Biology at Irkutsk State University.

### **Part 1. Fabrication of microcapsules**

Microcapsules carrying a fluorescent dye are prepared using a layer-by-layer assembly

of oppositely charged polyelectrolytes<sup>7,8</sup>. All procedures were performed at room temperature.

1.1 To synthesize porous CaCO<sub>3</sub> microcores enclosing the fluorescent dye, mix 2 mL of the SNARF-1-dextran solution (most polymer-bound fluorescent dye such as FITC-BSA can be used) at a concentration of ~2 mg/mL with 0.6 mL each of 1 mol/L solutions of CaCl<sub>2</sub> and Na<sub>2</sub>CO<sub>3</sub> under fast stirring.

Note: Pay attention to the different sensitivities of fluorescent dyes to photobleaching; if a light-sensitive fluorescent probe (like SNARF-1) is used, the manipulation and storage of the microparticles must be performed with as less light as possible.

1.2 After 5-10 s of agitation, transfer the suspension to 2 mL microcentrifuge tubes and centrifuge for 15 s at 10 000-12 000 g to pellet CaCO<sub>3</sub> microcores.

1.3 Discard the supernatant, wash the cores with ~2 mL of deionized water and resuspend the pellet by shaking.

1.4 Repeat the centrifugation-washing procedure three times in total. After the last centrifugation, discard the supernatant.

1.5 Incubate the microcores for 1 min in an ultrasonic bath to reduce their aggregation.

Note: CAUTION! Do not forget to protect ears with headphones.

1.6 To deposit the first polymeric layer on the templates, resuspend the cores in ~2 mL of a 4 mg/mL solution of poly(allylamine hydrochloride) (PAH) in 1 mol/L NaCl.

1.6.1 Keep the microcores in the solution for ~5 min with constant shaking.

1.6.2 After 15 s of centrifugation, discard the supernatant with the unbound PAH. Wash the covered microcores with deionized water at least 3 times through multiple centrifugation and washing steps. After the last centrifugation, discard the supernatant.

1.6.3. Incubate the microcores for 1 min in an ultrasonic bath to reduce their aggregation.

Note: If the applied fluorescent dye is cationic, start from poly(sodium 4-styrenesulfonate) (PSS) in 1 mol/L NaCl (see step #1.7).

1.7 Repeat step #1.6 with ~2 mL of a 4 mg/mL solution of PSS (also containing 1 mol/L NaCl) to deposit the second polymeric layer on the templates.

1.8 Repeat steps #1.6 and #1.7 six times to deposit 12 polymeric layers.

Note: It is not recommended to take a long break (~12 h or more) in the procedure until ~3-5 layers have been deposited because  $\text{CaCO}_3$  microcores without coverage may tend to recrystallize. Note that PSS coverage causes a higher aggregation of the microcores, and the long pause is advisable only when PAH or poly-L-lysine grafted with polyethylene glycol (PLL-g-PEG) is the outmost layer.

1.9 Incubate the covered microcores in 2 mg/mL PLL-g-PEG (~1 mL per microtube) for at least 2 h.

1.9.1 Wash the microcores with water via sequential centrifugation and resuspension steps. After the last centrifugation, discard the supernatant.

1.10 To obtain hollow microcapsules, dissolve the  $\text{CaCO}_3$  templates by adding 2 mL of 0.1 mol/L ethylenediaminetetraacetic acid (EDTA) solution (adjusted to pH 7.1 with NaOH) to the covered microcores.

1.10.1 After ~5 min of incubation, centrifugate the microcapsules for 45 s and discard the supernatant with the EDTA.

1.10.2 Repeat steps #1.10-1.10.1 twice.

1.11 Wash the microcapsules with 0.9% NaCl three times through multiple centrifugation steps within 45 s followed by washing steps. After the last centrifugation step, discard the supernatant.

Note: The final microcapsule solution for injection must be kept sterile (for example by adding ampicillin, 0.1 mg/mL), and the media should be biocompatible with the object of investigation (isotonic media with neutral pH).

1.12 Estimate concentration of the prepared microcapsules in a hemocytometer under a fluorescence microscope. Take a series of pictures of the microcapsules and measure the diameter of about a hundred microcapsules using ImageJ<sup>9</sup> or equivalent software and investigate the size distribution using the histogram.

1.13 Store the obtained encapsulated probe in the dark.

Note: After several washings in sterile 0.9% NaCl, the microcapsules can be stored for months at 4°C. Complete drying of the microcapsules during storage is not recommended.

## **Part 2. Preparation of optical setup and calibration of microencapsulated SNARF-1**

Rough pH measurements with microencapsulated SNARF-1 can be made using images in two channels of a fluorescent microscope<sup>7</sup>, but in this protocol a one-channel fluorescent microscope connected to a fiber spectrometer was applied.

2.1 Place the required set of fluorescence filters to the fluorescent microscope according to the characteristics of the applied fluorescent dye and turn on the fluorescent lamp.

2.1.1 Pull out the lever to the eyepieces.

Note: CAUTION! Excess light can damage the spectrometer matrix. Thus, make sure that the lever is in the "eyepiece" mode when the spectrometer is not used.

2.1.2 Connect one end of the optical fiber to the spectrometer and the other end to a collimator. Using adapters, place the collimator in the focus of the camera tube or other available port of the fluorescent microscope.

2.1.3 Turn on the spectrometer. Run the spectrometer control program and prepare the spectrometer for measurements.

2.2 For calibration of the microcapsule batch, place ~5 µL of the microcapsule suspension (~10 000 microcapsules per µL in deionized water) on a microscope slide, and dry the drop in a dark place (for example, in a thermostat at 35°C).

2.2.1 To calibrate the spectral characteristics of the microencapsulated SNARF-1, use a series of buffers with different pH values in range ~6-9. Drop ~10 µL of a buffer onto the dried microcapsules with SNARF-1-dextran and cover it with a coverslip.

2.2.2 Place the glass slide on the microscope stage. Locate the microcapsules using a ×40 objective.

2.2.3 Turn the microscope lever to the camera port. Register their fluorescence with the spectrometer. Turn the lever back to the eyepieces.

Note: Make sure the spectral signal is far beyond the background level, and ensure that the microcapsules in the field of view are not in a bubble (by switching to a lower magnification if necessary). Avoid prolonged illumination of the same microcapsules. SNARF-1 is sensitive to photobleaching.

2.2.4 Repeat step #2.2.3 for different microcapsules 10-15 times.

2.3 Calculate the fluorescence peak ratios (for example, using R or Scilab) for all registered spectra and determine the regression line between the median ratio (for each buffer) and medium pH using the following formula:

$$pH = a \log \left( \frac{I_{605}/I_{660} - 0.15}{1.1 - I_{605}/I_{660}} \right) + b$$

Note: SNARF-1 has a spectrum with two peaks corresponding to the emission of the protonated and deprotonated dye, and the ratio between the peaks is responsive to the pH of the medium. In this study, the ratio between the fluorescence intensity for 605 and 660 nm is used. These wavelengths are chosen depending on the filter set used.  $a$  and  $b$  are coefficients to be determined by non-linear regression (for example, using R). Values 0.15 and 1.1 are respectively the minimum and maximum values of  $I_{605}/I_{660}$

observed during the calibration.

2.4 Collect about 10  $\mu\text{L}$  of fish blood from approximately 5 adult animals. Place fish into a Petri dish with 1  $\mu\text{L}/\text{mL}$  water suspension of clove oil for anesthesia and wait until the animal turns on its side and stops responding to pinches of the fin (usually  $\sim 2\text{-}3$  min). Transfer the fish on a glass slide. Cut off the fish tail with a lancet and collect approximately 2  $\mu\text{L}$  of fish blood from the tail vein.

Note: To prevent blood clotting, treat the incision with heparin (5000 U/mL) and use heparinized glass capillaries and microcentrifuge tubes to collect the blood.

2.4.1 Drip about 10  $\mu\text{L}$  of blood with a pipette to the tip of the microelectrode and determine the pH using a pH-meter.

2.4.2 Drop the blood onto a slide with dried microcapsules and register the ratio of the fluorescence intensity as described for the calibration buffers (steps #2.2 -2.3).

2.5 Adjust the linear coefficient of the calibration curve to make the curve match the measurements in the fish blood (for more details see<sup>5</sup>).

### Part 3. Preparation for injection

3.1 Release the steel needle from the tip of the insulin pen (or syringe) by removing the plastic with a sharp lancet.

Note: Any thin needle ( $\varnothing$  0.33 mm or less) or glass capillary (usually  $\varnothing$  1 mm) can be prepared for microinjection<sup>10, 11</sup>.

3.2 Insert the needle halfway into the glass microcapillary; quickly and gently solder it using a gas torch.

3.3 Connect the glass microcapillary to the microinjector and flush it with sterile water three times. Ensure that the liquid flows through the needle.

3.4 Fill the system with distilled water.

Note: Make sure there are no bubbles in the system.

### Part 4. Injection

4.1 Take the prepared suspension of microcapsules in sterile 0.9% NaCl (or any other media used for the injections) with a concentration of 0.5 to 6 million microcapsules per microliter. Resuspend it using the ultrasonic bath for 1 min.

Note: Since the microcapsules tend to precipitate, during the following injection, shake the vial with the microcapsules mechanically (using a rotor) or manually every few minutes to resuspend them and prevent their aggregation.

4.2 Place the fish into a Petri dish with anesthetic (0.1 mL/L of clove oil suspended in water) for ~2-3 min. Wait until the fish turns on its side and stops responding to a light pinch of the fin.

4.3 Using a spoon, transfer the fish out of the anesthetic and gently place it on a damp sponge in a lateral position with the head towards the left (for right-handed person) or towards the right (for left-handed person).

4.4 Just before the injection, suck 1-2 mm of air into the glass capillary connected with microinjector. Then, load it with approximately 2  $\mu$ L of the dispersed microcapsules.

Note: Before the injection, the microcapsule solution must be adjusted to the temperature at which the fish are kept.

4.5 Gently stabilize the body of the fish on the sponge with non-dominant hand.

4.5.1 Find the lateral line of the fish. Mentally select a segment that extends from the operculum to the end of the abdominal cavity. Find the middle of this segment. Put the needle 1 mm lower in the ventral direction.

4.5.2 With a scraping movement, gently move the fish scales aside, and make a puncture. Insert the needle into the body at an angle of 45° to the table surface.

4.5.3 Push the needle toward the spine until it carefully rests against the spine.

4.5.4 Release approximately 1  $\mu$ L of the microcapsules' suspension into the kidney, and slowly withdraw the needle.

Note: To find the proper puncture site more easily, it is useful to practice finding the trunk kidney by transilluminating the fishes using a bottom light, as shown on Fig. 2A-B.

4.6 Rinse the fish from head to tail with a stream of water to remove any spilled microcapsules at the injection site.

## **Part 5. *In vivo* visualization**

5.1 Use the dissection scissors to remove the gill cover from the fish head, and denude the fish gills. Rinse the gills with water.

5.2 Using a spoon, transfer the fish to a microscope slide, and place it on the stage of the fluorescent microscope.

Note: Make sure that the gills of the fish do not dry out during successive procedures. To avoid this, periodically moisten them with water using a Pasteur pipette ([approximately every 1-2 min](#)).

5.3 Darken the room and using low magnification ( $\times 10$  objective) inspect the gills to find the fluorescent microcapsules.

Note: When the procedure is used for introduction of some fluorescent particles into fish circulatory system, it is recommended to inspect gills of several individuals for unexpected fluorescent particles before injections. Gills of wild-type zebrafish do not have autofluorescence, but in some cases sporadic fluorescent particles (like food pieces or unicellular symbionts) may be present on gills. If necessary, such particles can be recognized based on their specific shape (for example, food pieces have irregular shape, unlike spherical microcapsules) or fluorescence spectrum (i.e. color).

5.3.1 Switch the lens to a higher magnitude ( $\times 40$  objective), and position a microcapsule or a group of microcapsules in the center of the field of view.

5.3.2 Turn the lever to the port with a connected spectrometer. Record the spectral signal.

5.3.3 Turn the lever back to the eyepiece.

5.3.4 Repeat the measurements for different microcapsules several times.

5.4 Transfer the fish to the aquarium with proper aeration for recovery.

Note: With minimal practice, it is possible to perform the injection and signal recording at an approximate rate of 2-3 min per fish. The measurement can be repeated for one individual several times with the use of repeated low, harmless doses of anesthesia or another method of fixation. For long-term observation, use a system with continuous anesthesia<sup>12</sup>.

### **Representative results**

The obtained results refer to one of the three main categories of the presented protocol: the formation of fluorescent microparticles by encapsulation of a fluorescent dye (Fig. 1), the kidney injection of microcapsules with further visualization in gill capillaries (Fig. 2, 3) and, finally, the *in vivo* spectral recording of SNARF-1 fluorescence to monitor blood pH levels (Fig. 4).

The layer-by-layer approach using coating of the template  $\text{CaCO}_3$  cores enclosing the dye by multiple layers of oppositely charged polymers (PAH and PSS) and an outermost layer of a biocompatible polymer (PLL-g-PEG) is a simple and cost-effective method allowing to encapsulate different fluorescent probes such as SNARF-1-dextran, FITC-BSA or others (Fig. 1A). As a result, hollow microcapsules of micrometric size with stable semi-permeable elastic shells and loaded with the fluorescent dye are obtained (Fig. 1B). The microcapsules fabricated by this technique are typically non-uniform, with a normal distribution of particle size and certain median size in each batch (Fig. 1C).

**[Place Figure 1 here]**

**Figure 1: Layer-by-layer synthesis and characterization of hollow polyelectrolyte microcapsules loaded with a fluorescent dye. (A)** Scheme of a microcapsules assembly (drawn from a similar scheme published in<sup>4</sup>). **(B)** Picture of hollow microcapsules loaded with fluorescent dye FITC-BSA. **(C)** Size characterization of the batch of microcapsules from Figure 1B.

Robust and efficient microparticle implantation into the circulatory system of small fishes can be performed by manual injection directly into the fish kidney. The puncture in the proper site of the fish body (trunk kidney) is crucial for rapid delivery of microparticles by manual injection. The fish kidney is a hematopoietic organ and contains pigmented melanophores, and thus, it is well colored. Because it is nestled between transparent chambers of the swim bladder, it is easy to identify the organ in the intact animal with weak pigmentation or by transillumination of small fishes using a bottom light source, as shown on Fig. 2A-B.

[Place Figure 2 here]

**Figure 2: Localization of the proper site for kidney injection. (A)** Trans-illumination of the zebrafish demonstrates the localization of the trunk kidney (arrow). **(B)** Scheme illustrates how to identify the proper puncture site. The white dotted line indicates the lateral line of the abdominal segment of the fish. The arrow indicates the site for puncture and the proper direction of injection toward the spine. The swim bladder is designated by the green line. **(C)** Visualization of zebrafish kidney following the removal of organs and the body wall. An arrow points to the central bulge of the trunk kidney. **(D)** A sagittal histological section of *D. rerio* illustrates the general anatomy of the internal organs of the adult fish (H&E stain). **(E)** Micrograph of a fish kidney (dotted) scaled from (D) with an arrow pointing to the lumen of the posterior cardinal vein. Asterisks indicate the swim bladder.

To check the success of the injection procedure, a rapid visual inspection of the gills at low magnification ( $\times 10$ -20 objective) should be made after cutting the fish gill cover (Fig. 3A). Despite most of the microcapsules remain at the injection site or spill into the body cavity (Fig. 3B), if the injection is performed correctly, it is possible to observe the fluorescent microcapsules freely floating in the gill capillaries of the denuded fish gills immediately after the injection (Fig. 3C). If no fluorescent particles are detected in the gills, it is possible to repeat the injection in the same puncture. Successful delivery to the bloodstream should be obtained in approximately 70-90% of the total injected fish.

[Place Figure 3 here]

**Figure 3: Kidney injection of microcapsules with further visualization in gill capillaries. (A)** The overall scheme of the microcapsule delivery into the zebrafish bloodstream. **(B)** Fluorescent image of a zebrafish after the injection of the microcapsules with green FITC-BSA dye (the puncture site is indicated by an arrow). **(C)** This picture of the gills of fish, scaled from (B), demonstrates the successful delivery of microcapsules by kidney injection (gills of wild-type zebrafish have no autofluorescence).



During the injection directly into the fish trunk kidney, extensive bruising normally forms beneath the puncture site, indicating damage to the parenchyma capillaries or the renal vessels. There is no blood leakage out of the body because the puncture appears to quickly contract. Despite internal bleeding, individuals can still recover with approximately 80-90% surviving through the procedure (Table 1). It is also known that fish species can regenerate nephrons *de novo* after injury<sup>13, 14</sup>. If more than 20% of fish are dying, one must ensure that the anesthetization is performed properly.

[Place Table 1 here]

**Table 1. Safety and efficiency of the delivery of microcapsules into the zebrafish bloodstream.** The success of the procedure is determined by the presence of the fluorescent materials in the fish gill capillaries.

If the concentration of the injected microparticles is insufficient, too few particles may be available to be rapidly visualized in fish gills. At the same time, a suspension with a too high concentration can clog the needle. In case of layer-by-layer assembled microcapsules with median diameter ~2-5  $\mu\text{m}$ , concentration of approximately  $4 \cdot 10^5$ - $6 \cdot 10^6$  microcapsules per  $\mu\text{L}$  is optimal for effortless detection in fish gills after injection into fish kidney (Table 2).

[Place Table 2 here]

**Table 2. Record of the visual counting of FITC-BSA-containing microcapsules in *D. rerio* gills after injection (1.6  $\mu\text{L}$ ) into the trunk kidney.**

The proposed method of microparticles' implantation into fish circulatory system can be applied for *in vivo* monitoring of zebrafish blood pH by microencapsulated fluorescent probe, SNARF-1 (Fig. 4). SNARF-1 has a spectrum with two peaks corresponding to the emission of the protonated and deprotonated dye (Fig. 4A), thus the calibration curves of the ratio between the peaks at different pH of the media can be plotted (Fig. 4B). The components of the blood influence the readout of the encapsulated SNARF-1 (Fig. 4B), which can be evaluated experimentally by simultaneous measurement of blood pH by microcapsules and a pH-meter with a glass microelectrode. A putative calibration curve for microcapsules in zebrafish blood should be plotted by shifting the buffers' curve by the coefficient equal to the pH difference measured experimentally (see <sup>5</sup> for details).

Blood pH in zebrafish gill capillaries remains stable during at least several hours after the injection of microcapsules (Fig. 4C). At the same time, a short hypercapnic exposure leads to a statistically significant decrease of blood pH, which demonstrates applicability of the method for *in vivo* research on small fishes.

[Place Figure 4 here]

**Figure 4: Representative example of monitoring of zebrafish blood pH by registration of the fluorescence spectra of microencapsulated dye SNARF-1. (A)** Spectra of the prepared microcapsules loaded with SNARF-1 in sodium phosphate buffers at different pH. **(B)** Calibration curves of the prepared pH-sensitive microcapsules in sodium phosphate buffers and in extracted zebrafish blood. For all measurements, the mean $\pm$ s.d. is depicted.

(C) A representative example of the *in vivo* monitoring of zebrafish blood pH by encapsulated fluorescent SNARF-1 dye in zebrafish gill capillaries. In control conditions, blood pH remains stable during 4 hours after injection of microcapsules, while 5 minutes exposure under severe hypercapnia (900-1000 mg/L of dissolved CO<sub>2</sub>) causes acidification of the fish blood. Asterisk indicates statistically significant difference from the parallel control group with  $p < 0.01$  (Mann–Whitney U test).

## Discussion

To demonstrate the injection of microparticles into the zebrafish kidney, we used semi-permeable microcapsules loaded with an indicator dye. Thus, the protocol contains instructions for the fabrication of the microcapsules using the layer-by-layer assembly of oppositely charged polyelectrolytes<sup>7-8, 15-18</sup> (Fig. 1A). An advantage of this technology is that it is easy to perform with available laboratory equipment. Depending on the conditions and compounds used, the fabricated microcapsules can range from 0.5 to 100  $\mu\text{m}$  with nanometers-thick polyelectrolyte shell<sup>15</sup>. The synthesis parameters described in this manuscript result in elastic microcapsules consisting of 12 layers (in addition to the final biocompatible layer) of polymers approximately 2-6  $\mu\text{m}$  in size (Fig. 1B-C). The most important step determining the size of the microcapsules is the formation of the template microcores. This process involves the spontaneous formation of calcium carbonate crystals, and therefore, the obtained particles are non-uniform. Hence, a characterization of the microcapsule size distribution should be performed for every batch.

The important stage of this protocol is the optimization of the delivery of microparticles into the zebrafish circulatory system without the use of micromanipulation techniques. The administration of whole cells by retro-orbital injection has been previously described in detail in the Journal of Visualized Experiments<sup>3</sup>. However, in our experience, it is not easy for novice users to gain the injection efficiency described by the authors because the retro-orbital sinus is very small and located close to the pharynx and gill arches, which are easy to accidentally injure with a needle. Since less than a millimeter accuracy is required for the injections, the injecting properly is a rather difficult task. An equally quick and more efficient alternative (Table 1) is to inject directly into the fish kidney parenchyma. During the injection, the needle mechanically damages renal capillaries and blood vessels (e.g. the largest posterior cardinal vein), which allows entrance of microparticles into the circulatory system (Fig. 2E). Finally, the central bulge of the trunk kidney in adult zebrafish is large enough (up to 2 mm) for a manual injection without microsurgical equipment.

The proper positioning of the injection needle is critical for a successful manual administration. You can practice finding the trunk kidney in intact animals by transilluminating fishes using a bottom light, as shown in Fig. 2A-B. The scheme in Fig. 2B demonstrates how to properly perform the injection. If the procedure fails to administer microparticles into the blood stream, re-injection can be made at the same puncture within a short time frame with virtually no effect on the survival rate of the individuals.

Injection into the trunk kidney of adult zebrafish [\(also successfully tested on adult guppy \*Poecilia reticulata\*\)](#) is an effective method of microparticle delivery into the fish bloodstream with an allowable animal mortality; however, it is not perfectly suitable for drug administration due to variations in the injected volume. A weak point of this technique is a significant leakage of the solution out of the kidney into the abdomen because of the rapid administration. Nevertheless, a relative amount of the substance injected into bloodstream can be roughly estimated using visualization in the gill capillaries (Table 2).

There is no strict limitation of the injection volume, but the administration of more than 1  $\mu\text{L}$  of the suspension appears to be ineffective. The finest glass capillaries can be applied for the microinjection; however, because a large number of microcapsules tends to incite aggregation, we recommend the use of 31-29G ( $\varnothing$  0.33-0.25 mm) steel needles to avoid clogs in the needle lumen.

The success of microcapsule delivery through kidney injection into the bloodstream should be monitored using the rapid inspection for the presence of the fluorescent particles in the gills. Gills are easily accessible organs for *in vivo* observations. The gill filaments are a network of blood capillaries covered by a thin layer of respiratory epithelium, which makes intravital imaging of the blood flow in the gills particularly convenient (a kind of natural optical window). To perform the observation, the cartilage gill cover can be cut to expose the filaments. This procedure is safe for fish, which can live with denuded gills in well aerated water without a decrease in life expectancy. Moreover, the gills of large fish can be examined under a microscope simply by pushing the operculum out of the way<sup>19</sup>. In case of a successful injection, fluorescent microparticles immediately appear in the gills (Fig. 3). Note that implanted microparticles are considerably dissolved in the circulating blood volume, and a reduced number of particles reach the gill capillaries, thus sufficient concentration of microparticles should be selected for detection in fish gills after injection into fish kidney (Table 2).

The proposed procedure for implantation can be applied in a wide range of studies involving different species of small fishes. Despite the technique was developed and optimized for injection of fluorescent microparticles into circulatory system, it can also be applied for implantation of non-colored micro/nanoparticles or dissolved substances; however, in this case the effectiveness of injection should be verified in some other way. The currently described steps are optimal for such purposes as physiological monitoring using different optical micro-/nanosensors, visualization of vasculature, delivery of vaccines or drugs on some optically visible carriers and implantation of genetically modified cells.

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## Disclosures

The authors have nothing to disclose.

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MINISTRY OF EDUCATION AND SCIENCE OF THE RUSSIAN FEDERATION  
Federal State Budget Educational Institution  
of Higher Education  
"Irkutsk State University"

**RESEARCH INSTITUTE OF BIOLOGY**

3, Lenin str., Irkutsk, 664025 Russia

Tel.: +7(3952)24-30-77, fax: +7 (3952)34-00-07, e-mail: [director@bio.isu.ru](mailto:director@bio.isu.ru)

**Animal Subjects Research Committee**

Protocol No. 1

**Project title:** Monitoring of physiological parameters of fish blood using microencapsulated fluorescent probes

**Researcher's name:** Borvinskaya E.V., Gurkov A.N., Shchapova E.P.

**Supervisor:** Timofeev M.A.

**Department:** Laboratory of biosensors and ecophotonics

This letter certify that research "**Monitoring of physiological parameters of fish blood using microencapsulated fluorescent probes**" has been reviewed by the Animal Subjects Research Committee (ASRC) of the Institute of Biology at Irkutsk State University and has been approved from an ethical point of view. The study is allowed to be conducted in the presented form.

The ASRC expects to be informed about the any serious adverse events or significant change occurring in the course of the study and revision in the protocol which may alter its ethical consideration. In this case updated Application Form must be provided immediately.

The ASRC is working in accordance to Russian Federation legislation and policy requirements and also is strictly guided by EU Directive 2010/63/EU for studies involving animal subjects.

The following documents have been reviewed and approved:

Sr. No.	Name of the Documents	Date
1	Application Form	01.09.2017
2	Participant Information Sheet	01.09.2017

Chairperson of the Animal Subjects Research Committee

Silow E.A.

Date: 08.09.2017

