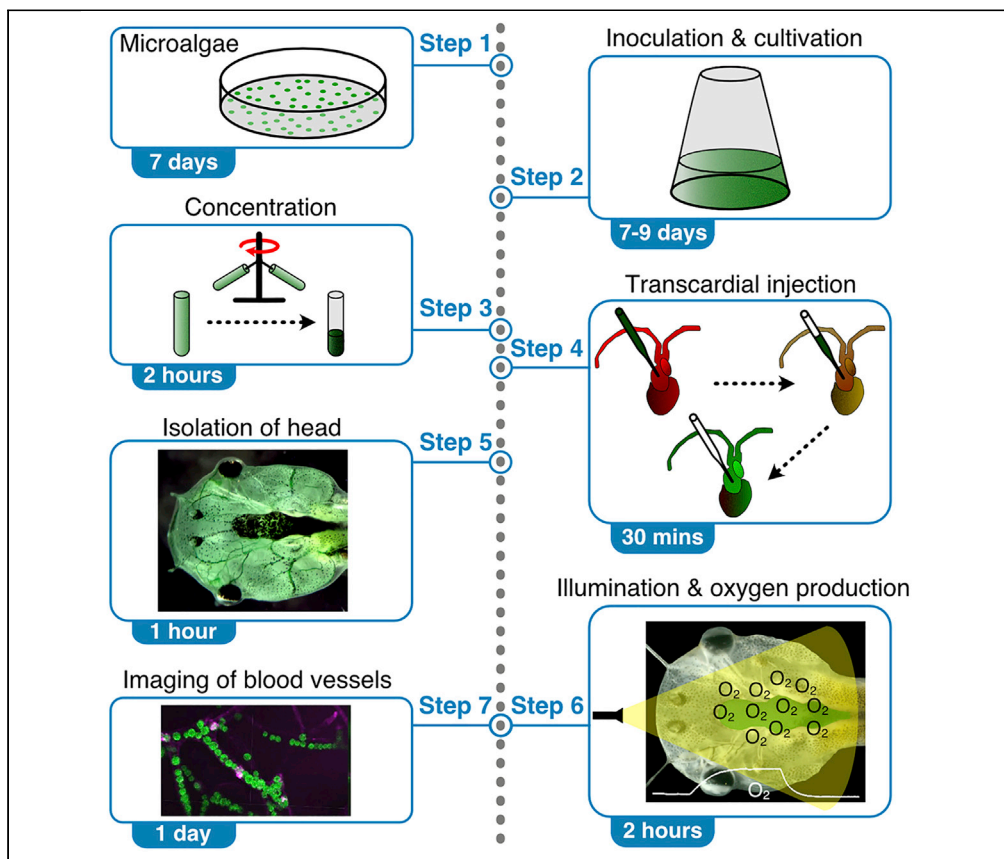


## Protocol

# Transcardial injection and vascular distribution of microalgae in *Xenopus laevis* as means to supply the brain with photosynthetic oxygen



Suzan Özugur, Myra N. Chávez, Rosario Sanchez-Gonzalez, Lars Kunz, Jörg Nickelsen, Hans Straka

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### Highlights

Transcardial injection of prokaryotic and eukaryotic microalgae in *Xenopus* tadpoles

Accumulation of microalgae in the brain through vascular distribution

Monitoring of  $O_2$  dynamics and neuronal activity in isolated whole-heads of tadpoles

Brain-wide photosynthetic  $O_2$  production by microalgae supports neurons

Oxygen in vertebrates is generally provided through respiratory organs and blood vessels. This protocol describes transcardial injection, vascular distribution, and accumulation of phototrophic microalgae in the brain of *Xenopus laevis* tadpoles. Following tissue isolation, oxygen dynamics and neuronal activity are recorded in semi-intact whole-head preparations. Illumination of such microalgae-filled preparations triggers the photosynthetic production of oxygen in the brain that, under hypoxic conditions, rescues neuronal activity. This technology is potentially able to ameliorate consequences of hypoxia under pathological conditions.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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## Protocol

Transcardial injection and vascular distribution of microalgae in *Xenopus laevis* as means to supply the brain with photosynthetic oxygen

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## SUMMARY

Oxygen in vertebrates is generally provided through respiratory organs and blood vessels. This protocol describes transcardial injection, vascular distribution, and accumulation of phototrophic microalgae in the brain of *Xenopus laevis* tadpoles. Following tissue isolation, oxygen dynamics and neuronal activity are recorded in semi-intact whole-head preparations. Illumination of such microalgae-filled preparations triggers the photosynthetic production of oxygen in the brain that, under hypoxic conditions, rescues neuronal activity. This technology is potentially able to ameliorate consequences of hypoxia under pathological conditions.

For complete details on the use and execution of this protocol, please refer to Özugur et al. (2021).

## BEFORE YOU BEGIN

The protocol describes the injection and distribution of phototrophic microalgae in the brain of *Xenopus laevis* tadpoles following transcardial injection of the microorganisms. Thus, before the injection, it is necessary to culture the microalgae and to concentrate the cells into a suspension prior to usage. The protocol will first provide a description of growth and proliferation of the employed microalgae followed by a list of key materials and equipment and continued by the different steps of the actual technical procedure. The study employs the microalgae *Chlamydomonas reinhardtii* (*C. reinhardtii*), a standard eukaryotic species in plant physiology (Figure 1A) and the cyanobacteria *Synechocystis* sp. PCC 6803 (*Synechocystis* 6803), a standard prokaryotic species used in photosynthesis research (Figure 1B).

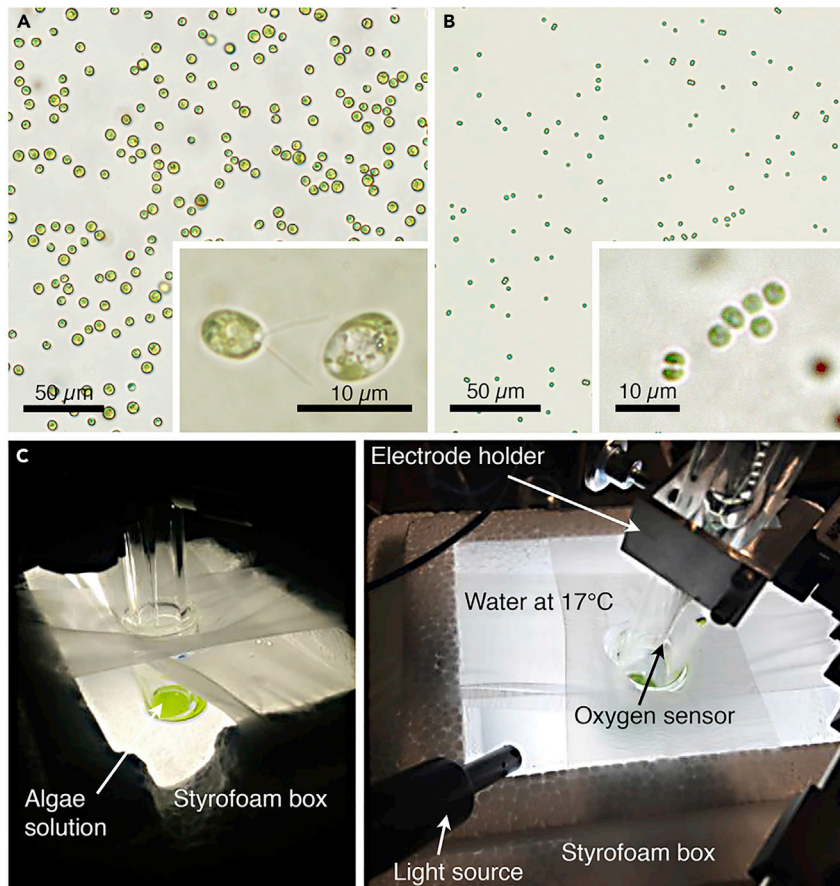
## Institutional permissions

All experiments on *Xenopus laevis* tadpoles were carried out in accordance with the ARRIVE guidelines and regulations. Permission for these experiments was granted by the legally responsible governmental institution (Regierung von Oberbayern) under the license codes ROB-55.2.2532.Vet\_03-17-24 and ROB-55.2.2532.Vet\_02-19-128. In addition, all experiments were performed in accordance with the relevant guidelines and regulations of the Ludwig-Maximilians-University Munich.

Growth and preparation of *C. reinhardtii*

© Timing: 7–8 days





**Figure 1. Eukaryotic green algae (*C. reinhardtii*) and prokaryotic cyanobacteria (*Synechocystis 6803*) produce oxygen upon illumination**

(A and B) Microphotographs depicting an overview and higher magnification (insets) of *C. reinhardtii* (A) and *Synechocystis 6803* (B).

(C) Setup for measurements of photosynthetic  $O_2$  production upon illumination in isolated suspensions of microorganisms.

(A–C) adapted from and Özugur (2021) and Özugur et al. (2021), with permission under the CC BY-NC-ND license agreement.

This major step describes the culturing and preparatory steps that are required to produce a suspension of *C. reinhardtii* that can be further concentrated shortly before the transcatheter injection.

1. Prepare Tris acetate phosphate (TAP) agar plates and TAP-medium supplemented with sorbitol.
2. Grow stable colonies of the cell-wall deficient, arginine phototropic, cw15-30-derived UVM11 *C. reinhardtii* strain or the photomutant *nac2-26* strain of *C. reinhardtii* (Boudreau et al., 2000) on TAP-agar plates.
3. Start a *C. reinhardtii* pre-culture by inoculating 100 mL TAPS (TAP-medium supplemented with 1% weight/volume sorbitol) medium with plate-growing algae.
4. Grow the algae in an orbital shaker at 20°C and under constant illumination ( $30 \mu E \cdot m^{-2} \cdot s^{-1}$ ) and agitation (120 rpm).
5. At the middle log-phase, e.g., when the algae suspension reaches a cell-density of about  $0.5 \cdot 10^7$  cells  $\cdot mL^{-1}$ , transfer the algae suspension into 1-liter medium in an Erlenmeyer flask and grow the culture under constant illumination ( $30 \mu E \cdot m^{-2} \cdot s^{-1}$ ) and agitation (120 rpm) for 4–5 days to reach a final cell-concentration of  $10^7$  cells  $\cdot mL^{-1}$ .
6. Use a Neubauer-chamber to systematically determine the cell concentration under a microscope.

**Note:** Optimal growth conditions for these photomixotrophic algae are at 20°C and continuous illumination (30  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ). Also, the *nac2-26* strain lacks the nuclear *Nac2* gene for stable accumulation of *psbD* mRNA, encoding the D2 reaction center polypeptide of the photosystem and thus renders this strain incapable of photosynthesis. It is, thus, important to transfer the colonies onto fresh plates at least once a month to maintain a healthy green color and growth capacity.

### Growth and preparation of *Synechocystis* 6803

⌚ Timing: 6–7 days

This major step describes the culturing and preparatory steps that are required to produce a suspension of *Synechocystis* 6803 that can be further concentrated shortly before the transcatheter injection.

7. Prepare BG11 agar plates and medium following the instructions in the [materials and equipment](#) section.
8. Grow stable colonies of the wild-type cyanobacteria *Synechocystis* 6803 strain and its photomutant TD4122 on BG11 agar plates supplemented with 5  $\text{mmol} \cdot \text{L}^{-1}$  glucose, at 30  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$  and 28°C (Nixon et al., 1992).
9. Start a 50 mL preculture by inoculating *Synechocystis* 6803 cyanobacteria in BG11 medium supplemented with 5  $\text{mmol} \cdot \text{L}^{-1}$  glucose.
10. Grow the culture for 2–3 days at 30°C under constant illumination (30–50  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) and agitation (120 rpm).
11. Determine the cell density of the preculture by measuring the absorbance in a spectrophotometer (Nanophotometer P330, Implen GmbH).
12. Use the preculture to inoculate two 0.75-liter cultures at an  $\text{OD}_{750} = 0.01$ , which is equivalent to  $2 \cdot 10^5$   $\text{cells} \cdot \text{mL}^{-1}$ .
13. Grow the cyanobacteria culture for 3–4 days at 30°C under constant aeration and illumination (30  $\mu\text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ ) until an  $\text{OD}_{750} \geq 2$  is reached.

**Note:** The TD4122 strain lacks the three copies of the *psbA* gene encoding the photosystem II subunit D1, thus rendering it incapable of photosynthetic oxygen production. Thus, plate cyanobacteria colonies on fresh plates once a month to maintain the colonies in good health and ensure optimal growth.

⚠ **CRITICAL:** Algal and cyanobacterial cell-suspensions have to be handled gently to avoid cell damage. Also, the cells need to be fully resuspended to avoid clogging of the injection capillary during transcatheter pressure injection. See [troubleshooting 1](#).

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
NaCl	Carl Roth	3957.1
NaHCO <sub>3</sub>	Carl Roth	8551.1
KCl	Carl Roth	P017.1
D(+)-Glucose monohydrate for frog Ringer	Carl Roth	6780.1
Hepes	Carl Roth	9105.2
CaCl <sub>2</sub>	Carl Roth	T885.2
MgCl <sub>2</sub>	Carl Roth	2189.2
NaOH	Carl Roth	K021.1
HCl	Carl Roth	K.025.1
Ascorbic acid	Carl Roth	3525.3

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<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Na <sub>2</sub> HPO <sub>4</sub> · 2H <sub>2</sub> O	Carl Roth	4984
KH <sub>2</sub> PO <sub>4</sub>	Merck (Sigma-Aldrich)	1.05108
CaCl <sub>2</sub> · 2 H <sub>2</sub> O	Carl Roth	HNO4.3
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	Merck (Sigma-Aldrich)	105886
NH <sub>4</sub> Cl	Carl Roth	K.298
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> · 4 H <sub>2</sub> O	Carl Roth	3.666
CoCl <sub>2</sub> · 6 H <sub>2</sub> O	Merck (Sigma-Aldrich)	C8661
CuSO <sub>4</sub> · 5 H <sub>2</sub> O	Carl Roth	8175
FeSO <sub>4</sub> · 7 H <sub>2</sub> O	Carl Roth	3722
H <sub>3</sub> BO <sub>3</sub>	Merck (Sigma-Aldrich)	B6768
MnCl <sub>2</sub> · 4 H <sub>2</sub> O	Merck (Sigma-Aldrich)	244589
Na <sub>2</sub> EDTA · 2 H <sub>2</sub> O	Carl Roth	8043
ZnSO <sub>4</sub> · 7 H <sub>2</sub> O	Carl Roth	K301
KOH	Carl Roth	P747
KPO <sub>4</sub>	Carl Roth	3904
Tris	Carl Roth	4855
D-Sorbitol	Carl Roth	6213
Agar	Carl Roth	5210
Citric acid · H <sub>2</sub> O	Zefa	Z-0625
NaNO <sub>3</sub>	Carl Roth	8601
Co(NO <sub>3</sub> ) <sub>2</sub>	Merck (Sigma-Aldrich)	203106
Na <sub>2</sub> MoO <sub>4</sub> · 2 H <sub>2</sub> O	Merck (Sigma-Aldrich)	M1003
K <sub>2</sub> HPO <sub>4</sub>	Merck (Sigma-Aldrich)	1.05109
Na <sub>2</sub> CO <sub>3</sub>	Carl Roth	8563
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> · 5 H <sub>2</sub> O	Carl Roth	8649
FeNH <sub>4</sub> citrate	Merck (Sigma-Aldrich)	W527106
α-D-Glucose monohydrate for algae growth	Carl Roth	6887
TES-KOH pH 8.2	Carl Roth	9137
<b>Experimental models: Organisms/strains</b>		
<i>Xenopus laevis</i> wildtype, developmental stage 52–53 (Nieuwkoop and Faber, 1994), both sexes	Institutional breeding facility, Faculty of Biology, LMU Munich	N/A
<i>Chlamydomonas reinhardtii</i> wildtype	Faculty of Biology, LMU Munich	Neupert et al. (2009)
<i>Chlamydomonas reinhardtii</i> nac2-26 strain	Faculty of Biology, LMU Munich	Boudreau et al. (2000)
Cyanobacteria <i>Synechocystis</i> 6803 wildtype	Faculty of Biology, LMU Munich	N/A
Cyanobacteria <i>Synechocystis</i> 6803 TD41 strain	Faculty of Biology, LMU Munich	Nixon et al. (1992)
<b>Software and algorithms</b>		
Spike 2 version 7.04	Cambridge Electric Design Limited (CED), UK	N/A
Microcal Origin 6.0G	OriginLab Corp., USA	N/A
Affinity Designer 1.8.3	Serif Europe, UK	N/A
<b>Other</b>		
Sorvall™ RC6Plus	Fisher Scientific	N/A
Nanophotometer P330	Implen GmbH	N/A
Stereomicroscope (SZX7)	Olympus	N/A
Pressure applicator FemtoJet 4i	Eppendorf	N/A
Pressure applicator	NPI Electronic	PDES-02T
Pulse master A300	World Precision Instruments	N/A
Electrode puller (P-87 Brown)	SUTTER INSTRUMENT	N/A
Micropipette Grinder EG-45	NARISHIGE	N/A
Light source (CL 6000 LED)	Zeiss	N/A
Mavig VivaScope RS-G4	Vivascope/Mavig	N/A
Oxygen-sensitive microsensors	Unisense A/S	N/A

(Continued on next page)

### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Amplifier	NPI Electronic	EXT 10-2F
Micromanipulator	Sensapex	N/A
A/D board (CED 1401)	Cambridge Electric Design Limited (CED)	N/A

## MATERIALS AND EQUIPMENT

### Frog Ringer solution

Frog Ringer solution is made from a 10× stock solution and CaCl<sub>2</sub> and MgCl<sub>2</sub> stock solutions.

#### Frog Ringer 10× stock solution

Reagent	Final concentration	Amount
NaCl	750 mmol · L <sup>-1</sup>	40.38 g
NaHCO <sub>3</sub>	250 mmol · L <sup>-1</sup>	20.10 g
KCl	20 mmol · L <sup>-1</sup>	1.50 g
Glucose	110 mmol · L <sup>-1</sup>	20.18 g
Hepes	100 mmol · L <sup>-1</sup>	20.38 g
ddH <sub>2</sub> O	n/a	1,000 mL

Store at 4°C until use or for a maximal period of 7 days.

#### CaCl<sub>2</sub> stock solution

Reagent	Final concentration	Amount
CaCl <sub>2</sub>	200 mmol · L <sup>-1</sup>	2.94 g
ddH <sub>2</sub> O	n/a	100 mL

Store at 4°C until use or for a maximal period of 7 days.

#### MgCl<sub>2</sub> stock solution

Reagent	Final concentration	Amount
MgCl <sub>2</sub>	200 mmol · L <sup>-1</sup>	4.06 g
ddH <sub>2</sub> O	n/a	100 mL

Store at 4°C until use or for a maximal period of 7 days.

#### Frog Ringer (1×) made from stock solutions (see above)

Reagent	Final concentration	Amount
Frog Ringer 10× stock solution (see above)	n/a	100 mL
CaCl <sub>2</sub> stock solution (see above)	2 mmol · L <sup>-1</sup>	10 mL
MgCl <sub>2</sub> stock solution (see above)	0.5 mmol · L <sup>-1</sup>	2.5 mL
ddH <sub>2</sub> O	n/a	887.5 mL

Oxygenize with carbogen (95% O<sub>2</sub>, 5% CO<sub>2</sub>) at 20°C until use or for maximally for 1 day.

## Stock solutions for TAP medium and agar plates

#### Phosphate buffer 10× stock solution

Reagent	Final concentration	Amount
Na <sub>2</sub> HPO <sub>4</sub> · 2H <sub>2</sub> O	66 mmol · L <sup>-1</sup>	58.75 g
KH <sub>2</sub> PO <sub>4</sub>	15 mmol · L <sup>-1</sup>	10 g
NaCl	136.9 mmol · L <sup>-1</sup>	400 g
KCl	27 mmol · L <sup>-1</sup>	10 g
ddH <sub>2</sub> O	n/a	5,000 mL

Autoclave and store at 4°C for up to 2 months.

#### Beijerinck salt 40× stock solution

Reagent	Final concentration	Amount
CaCl <sub>2</sub> · 2 H <sub>2</sub> O	14 mmol · L <sup>-1</sup>	2 g
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	16 mmol · L <sup>-1</sup>	4 g
NH <sub>4</sub> Cl	299 mmol · L <sup>-1</sup>	16 g
ddH <sub>2</sub> O	n/a	1,000 mL

Autoclave and store at 4°C for up to 2 months.

#### Cold trace elements for *Chlamydomonas* 1,000× stock solution

Reagent	Final concentration	Amount
(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> · 4 H <sub>2</sub> O	0.9 mmol · L <sup>-1</sup>	1.1 g
CoCl <sub>2</sub> · 6 H <sub>2</sub> O	6.8 mmol · L <sup>-1</sup>	1.61 g
CuSO <sub>4</sub> · 5 H <sub>2</sub> O	6.3 mmol · L <sup>-1</sup>	1.57 g
FeSO <sub>4</sub> · 7 H <sub>2</sub> O	17.9 mmol · L <sup>-1</sup>	4.99 g
H <sub>3</sub> BO <sub>3</sub>	184.4 mmol · L <sup>-1</sup>	11.4 g
MnCl <sub>2</sub> · 4 H <sub>2</sub> O	25.6 mmol · L <sup>-1</sup>	5.06 g
Na <sub>2</sub> EDTA	148.7 mmol · L <sup>-1</sup>	50 g
ZnSO <sub>4</sub> · 7 H <sub>2</sub> O	76.5 mmol · L <sup>-1</sup>	22 g
ddH <sub>2</sub> O	n/a	1,000 mL

Adjust to pH 5.5 with KOH; prepare as in Hill and Kafer (2001); store aliquots at -20°C for long-term or at 4°C for up to 6 months.

#### TAP 40× stock solution

Reagent	Final concentration	Amount
KPO <sub>4</sub>	40 mmol · L <sup>-1</sup>	40 mL
Tris	800 mmol · L <sup>-1</sup>	96.8 g
ddH <sub>2</sub> O	n/a	1,000 mL

Adjust to pH 7 with acetic acid (> 40 mL), autoclave and store at 4°C for up to 3 months.

### TAP-medium and agar plates

#### TAP- liquid medium

Reagent	Final concentration	Amount
Beijerinck salt 40× stock solution (see above)	n/a	25 mL
Cold trace elements for <i>Chlamydomonas</i> 1,000× (see above)	n/a	1 mL
TAP 40× stock solution (see above)	n/a	25 mL
ddH <sub>2</sub> O	n/a	1,000 mL

Adjust to pH 7 with acetic acid or KOH, autoclave and store at 20°C for no longer than 1 month.

#### TAP-agar plates

Reagent	Final concentration	Amount
Agar	n/a	18 g
Beijerinck salt 40× stock solution (see above)	n/a	25 mL
Cold trace elements for <i>Chlamydomonas</i> 1,000× (see above)	n/a	1 mL
TAP 40× stock solution (see above)	n/a	25 mL
ddH <sub>2</sub> O	n/a	1,000 mL

Adjust to pH 7 with acetic acid or KOH before adding the agar, autoclave and store at 20°C for no longer than 1 month.

**BG11-medium and agar plates**

**Trace Elements for *Synechocystis* (1,000×)**

Reagent	Final concentration	Amount
Co(NO <sub>3</sub> ) <sub>2</sub> · 6 H <sub>2</sub> O	0.17 mmol · L <sup>-1</sup>	0.05 g
CuSO <sub>4</sub> · 5 H <sub>2</sub> O	0.32 mmol · L <sup>-1</sup>	0.08 g
H <sub>3</sub> BO <sub>3</sub>	46.3 mmol · L <sup>-1</sup>	2.86 g
MnCl <sub>2</sub> · 4 H <sub>2</sub> O	9.15 mmol · L <sup>-1</sup>	1.81 g
Na <sub>2</sub> MoO <sub>4</sub> · 2 H <sub>2</sub> O	1.61 mmol · L <sup>-1</sup>	0.39 g
ZnSO <sub>4</sub> · 7 H <sub>2</sub> O	0.77 mmol · L <sup>-1</sup>	0.22 g
ddH <sub>2</sub> O	n/a	1,000 mL

Sterile-filter and store at 4°C for up to 6 months.

**BG-FPC (100×)**

Reagent	Final concentration	Amount
CaCl <sub>2</sub> · 2 H <sub>2</sub> O	24.5 mmol · L <sup>-1</sup>	1.8 g
Citric acid · H <sub>2</sub> O	3.12 mmol · L <sup>-1</sup>	0.328 g
MgSO <sub>4</sub> · 7 H <sub>2</sub> O	30.4 mmol · L <sup>-1</sup>	3.8 g
Na-EDTA · 2 H <sub>2</sub> O	0.279 mmol · L <sup>-1</sup>	0.052 g
NaNO <sub>3</sub>	1,760 mmol · L <sup>-1</sup>	74.8 g
Trace Elements for <i>Synechocystis</i> (1,000×) see above	n/a	50 mL
ddH <sub>2</sub> O	n/a	500 mL

Autoclave and store at 4°C for up to 3 months.

**BG11-liquid medium**

Reagent	Final concentration	Amount
BG-FPC (100×) see above	n/a	10 mL
FeNH <sub>4</sub> citrate	25 μmol · L <sup>-1</sup>	1 mL
Glucose	5 mmol · L <sup>-1</sup>	5 mL
K <sub>2</sub> HPO <sub>4</sub> <sup>a</sup>	175 μmol · L <sup>-1</sup>	1 mL
Na <sub>2</sub> CO <sub>3</sub>	189 μmol · L <sup>-1</sup>	1 mL
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> · 5 H <sub>2</sub> O	19 mmol · L <sup>-1</sup>	4.7 g
ddH <sub>2</sub> O	n/a	1,000 mL

<sup>a</sup> Add sterile K<sub>2</sub>HPO<sub>4</sub> 175 mmol · L<sup>-1</sup> stock to the autoclaved BG11 medium. Store at 20°C for no longer than 1 month.

**BG11-agar plates**

Reagent	Final concentration	Amount
Agar	n/a	15 g
BG-FPC (100×) see above	n/a	10 mL
FeNH <sub>4</sub> citrate	25 μmol · L <sup>-1</sup>	1 mL
Glucose	5 mmol · L <sup>-1</sup>	5 mL
K <sub>2</sub> HPO <sub>4</sub> <sup>a</sup>	175 μmol · L <sup>-1</sup>	1 mL
Na <sub>2</sub> CO <sub>3</sub>	189 μmol · L <sup>-1</sup>	1 mL
Na <sub>2</sub> S <sub>2</sub> O <sub>3</sub> · 5 H <sub>2</sub> O	19 mmol · L <sup>-1</sup>	4.7 g
TES-KOH pH 8.2	1,000 mmol · L <sup>-1</sup>	10 mL
ddH <sub>2</sub> O	n/a	1,000 mL

<sup>a</sup> Add sterile K<sub>2</sub>HPO<sub>4</sub> 175 mmol · L<sup>-1</sup> stock to the autoclaved BG11 medium and then pour the plates. Store at 4°C for up to 1 month.

**Note on storage conditions:** FeNH<sub>4</sub> citrate, Glucose, K<sub>2</sub>HPO<sub>4</sub>, Na<sub>2</sub>CO<sub>3</sub> and TES-KOH stock solutions should be autoclaved and then be stored at 4°C for up to 6 months.



## STEP-BY-STEP METHOD DETAILS

### Testing the oxygen production capacity of phototrophic microorganisms in isolation

⌚ Timing: 1–2 h

This major step describes the procedure to test the oxygen production capacity of the microorganisms in isolation before employment in the transcatheter injections.

1. Provision of algal suspensions for measurements of the oxygen production capacity.
  - a. For cyanobacteria, bring the culture to the required cell-density of  $10^{10}$  cells · mL<sup>-1</sup> by step-wise centrifugation (5 min, 3,000 g, 20°C).
  - b. For *C. reinhardtii*, bring the culture to the required cell-density of  $10^{10}$  cells · mL<sup>-1</sup> by step-wise centrifugation (5 min, 150–290 g, 20°C).
2. Oxygen production in isolated suspensions of phototrophic microorganisms.
  - a. Prepare a Styrofoam box with water at 17°C (Figure 1C).
  - b. Insert a thermometer and maintain the ambient temperature at 17°C using ice or warm water.
  - c. Solidly mount a tripod with a micromanipulator on a base plate next to the box.
  - d. Mount an O<sub>2</sub>-sensitive microsensors (Unisense A/S, Denmark) with tip diameter of 10 μm onto the micromanipulator for the advancement of the electrode (Özugur, 2021).
  - e. Suspend 0.1 or  $0.5 \times 10^9$  cells · mL<sup>-1</sup> in 5 mL frog Ringer solution and fill into a tube with a diameter of 3 cm (Figure 1C).
  - f. Place and firmly secure the tube containing the microalgae suspension inside the Styrofoam box.
  - g. Mount a tripod with an adjustable light source (Zeiss CL 6000 LED, with > 150 W, 600 lm) next to the Styrofoam box and place the end of the flexible fiber optics at a distance of 5 cm from the tube with sample (Figure 1C).
  - h. Set the light intensity to 100 μE.
  - i. Insert the O<sub>2</sub>-sensitive microsensors into the suspension and maintain the algae for 30 min in darkness to determine the baseline level in the absence of light.
  - j. Illuminate the isolated algae suspension in the tube and record the gradual change in O<sub>2</sub> concentration until a steady-state level is reached.
  - k. The O<sub>2</sub> concentration for either type of microalgae should be in the range of 2,000–4,000 μmol/l (see Özugur, 2021).

### Preparatory steps prior to the transcatheter injection of microalgae

⌚ Timing: 2 days (step 3)

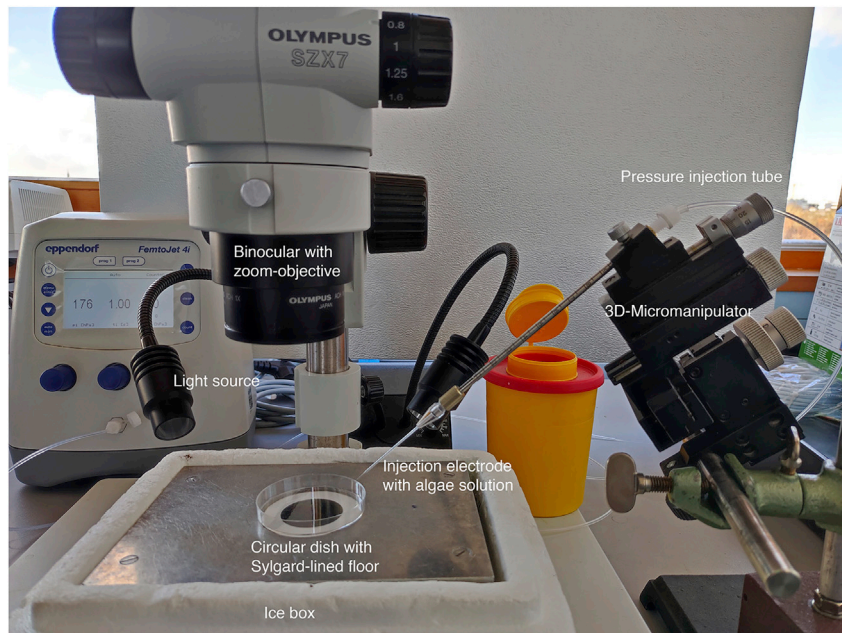
⌚ Timing: 20–30 min (step 4)

⌚ Timing: 30 min (step 5)

⌚ Timing: 1 h (step 6)

This major step describes the preparation of tools and solutions that are required for the actual transcatheter injection of microalgae.

3. Preparation of the container for the injection.
  - a. Prepare a circular dish (diameter: 5.5 cm) by cleaning with 70% ethanol.
  - b. Mix Silicone Elastomer base (Sylgard, Dow; Catalog number, 184) with curing agent (10:1) by gently stirring the compound for 2–3 min.
  - c. Pour the viscous Sylgard compound into the circular dish up to a height of ~3 mm.



**Figure 2. Setup for transcordial injections of algae suspensions into the vascular system of *Xenopus laevis* tadpoles**  
The spatial arrangement of the binocular, micromanipulator, light source and chamber, holding the animals during the injection have been optimized for visibility and accessibility of the tissue for the injection.

- d. Cover the dish to protect from dust and place it horizontally for 1–2 days at 20°C until the compound has become solid.

**Note:** Sylgard-filled dishes can be multiply used and stored under dry, dust-free conditions for several months.

4. Production of glass capillaries for the injection.
  - a. For the injection, use microcapillaries made from borosilicate glass (diameter: 1.5 mm, length 10 cm, GB150-8P, Science Products, Hofheim, Germany).
  - b. Pull microcapillaries on a horizontal puller (e.g., P-87 Brown/Flaming electrode puller).
  - c. Break the tips of the microcapillaries with fine forceps under a binocular to reach an outer diameter of 50–100  $\mu\text{m}$ .
  - d. Insert microcapillaries on a rotating grinder (e.g., Micropipette Grinder EG-45, Narashige) at an angle of 30° and gently grind the tip to obtain an injection needle-like sharp surface.
  - e. Make 5–6 microcapillaries to have sufficient substitutes available for the injection.

**Note:** After grinding, microcapillaries can be stored in a dry, dust-free container for several weeks.

5. Assembly of the micromanipulator-guided pressure injection device (Figure 2).
  - a. Prepare a plain and clean metal support plate of about 50 × 40 cm (e.g., magnetic or with drill holes) underneath a binocular (e.g., Olympus SZX7) with a zoomable lens (2.5× to 20×).
  - b. Solidly mount a tripod with a 3-axes micromanipulator (e.g., Bachhofer, Reutlingen) on a base plate next to or on the main plate.
  - c. Attach a holder for electrodes with a diameter of 1.5 mm, equipped with a connector for applying pressurized air onto the micromanipulator (Figure 2).

- d. Fix an incompressible polyethylene tube (Inner diameter: 2 mm) to the electrode holder and connect with a pressure injection device (e.g., PDES-02T; npi electronics, Tamm, Germany or FemtoJet 4i; Eppendorf, Hamburg, Germany).
- e. Connect the pressure injection device (PDES-02T) with a BNC cable to an electrical pulse generator (e.g., Pulse master A300; World Precision Instruments, Sarasota, USA) equipped with a remote control to individually trigger single pulses.
- f. Adjust the pressure magnitude to 1 bar and the pulse duration to 100 ms.
- g. Place a container (20 × 10 × 5 cm), covered with a flat surface in the center of the base plate (see above) underneath the binocular (Figure 2); the container (ice box) has to be filled with crushed ice prior to the injection procedure (see below).
- h. Place a fiber optics adjacent to the setup to provide appropriate illumination for the surgery and injection.

**Note:** Any pressure injection device will suffice for the procedure as long as the injector allows providing short, temporally modifiable pulses in the range of 50–500 ms and a pressure of 0.5–2 bar.

6. Provision of algal suspensions for transcardial injections.
  - a. For cyanobacteria, bring the culture to the required cell-density of  $10^{10}$  cells · mL<sup>-1</sup> by step-wise centrifugation (see above, 5 min, 3,000 g, 20°C).
  - b. For *C. reinhardtii*, bring the culture to the required cell-density of  $10^{10}$  cells · mL<sup>-1</sup> by step-wise centrifugation (see above, 5 min, 150–290 g, 20°C).
  - c. Resuspend the algae and cyanobacteria in frog Ringer solution at the target concentration in a small lockable container, enwrapped with aluminum foil to prevent excessive light exposure.

**Note:** 5 mL of concentrated suspension are sufficient for easy handling and multiple refills of the injection microcapillaries.

### Transcardial injection of microalgae in *Xenopus laevis* tadpoles

⌚ Timing: 1 h (step 7)

⌚ Timing: 1 h (step 8)

⌚ Timing: 15 min (step 9)

⌚ Timing: 10 min (step 10)

⌚ Timing: 30 min (step 11)

This major step describes the surgery of *Xenopus* tadpoles to access the heart and the actual transcardial injection of the suspension of microalgae.

7. Preparation of the anesthetics.
  - a. Freshly prepare the anesthetics by dissolving 3-aminobenzoic acid ethyl ester methanesulfonate (MS-222; Pharmaq Ltd., UK) in a volume of 50 mL cold frog Ringer solution.
  - b. The anesthetics can be stored at 4°C in darkness for up to two weeks.
8. Preparation of the fixative.
  - a. Freshly prepare paraformaldehyde (PFA) fixative, by adding PFA powder to 50 mL of 0.1 M · L<sup>-1</sup> phosphate buffer.
  - b. Gently stir the solution on a heat plate at 62°C.
  - c. Add a few drops of 1 N NaOH to completely clear the solution.

d. Cool down the PFA fixative to 20°C and adjust the pH with HCl to 7.3–7.4 before usage.

**Note:** The preparation of the PFA solution has to be performed under a hood to meet the safety instructions for the handling of this compound.

### 9. Anesthesia of mid-metamorphic tadpoles.

- Place the tadpole in the 0.05% MS-222 solution until the animal is deeply anesthetized (lack of a tail pinch-induced reflex), which takes about 5–10 min.
- Transfer the anesthetized tadpole into the Sylgard-lined circular dish (diameter: 5 cm), filled with cold (~10°C) frog Ringer solution and 0.05% MS-222 to maintain the deep anesthesia throughout the injection procedure.
- Mechanically secure the anesthetized tadpole at the level of the rostral tail and the head ventral side up with U-shaped pins to the floor of the Sylgard-lined dish.

**Note:** The procedure here is described for *Xenopus laevis* tadpoles at developmental stages 52–53 (Nieuwkoop and Faber, 1994). Therefore, indicated times and volumes have to be individually adjusted when using younger (smaller) or older (larger) larvae. The mechanically secured tadpoles must be completely submerged and covered by frog Ringer solution for at least 1–2 mm above the tissue.

### 10. Pre-injection surgery.

- Fill the container underneath the binocular with crushed ice to ensure a continuous cooling of the frog Ringer solution during the injection procedure (Figure 2).
- Place and firmly fix the Sylgard-lined dish with the anesthetized and mechanically secured tadpole onto the flat surface above the container (Figure 2).
- Use the fiber optics to illuminate the preparation and to optimize the visibility of the tissue (Figure 2).
- Gently remove the skin above the heart with a microscissor and forceps, such that the beating heart becomes freely accessible.

**Note:** Avoid excessive amounts of light to prevent light-stimulation of the algae.

### 11. Injection of the microalgae.

- For physiological experiments:** Open the container with the concentrated suspension of microalgae and fill 10  $\mu$ L of the algae suspension with a 10  $\mu$ L pipette into a long 20  $\mu$ L tip without air bubbles. See [troubleshooting 2](#).
- For imaging experiments of algae inside blood vessels:** Mix 8  $\mu$ L algae suspension with 2  $\mu$ L of Isolectin GS-IB4 obtained from *Griffonia simplicifolia* Alexa Fluor™ 488 conjugate and fill the solution bubble-free into the microcapillary.
- Insert the microcapillary into the electrode holder, mounted onto the 3-axes micromanipulator.
- Advance the microcapillary until entering the Ringer solution above the tadpole and position the tip close to the surface of the beating heart.
- Gently hold the beating heart steady with fine forceps, guide and insert the microcapillary with the 3-axes micromanipulator into the ventricular chamber of the heart.
- Let the algae-filled microcapillary recover inside the heart for 1 min.
- Manually trigger single pulses (100 ms, 1 bar) with the remote control in synchrony with the heartbeat ([Methods video S1](#)).
- If no algae are injected, increase the pulse duration up to 200 ms and/or the pressure up to 2 bar; if this is still unsuccessful, exchange the microcapillary for another one with a larger diameter. See [troubleshooting 3](#).

- i. Repeat (~5 pulses per minute) until the volume of 10  $\mu\text{L}$  has been injected; each pulse injects an approximate volume of 0.1–0.2  $\mu\text{L}$ .
- j. The injection of air into the heart with the last pulse must be avoided.
- k. After the last pulse, gently remove the microcapillary and let the tadpole recover for 5 min before continuing the procedure with the euthanasia of the animal.

△ **CRITICAL:** The success of the injection becomes apparent by the pulsed ejection of the microalgae into the bilateral aortic arches (Methods video S1) and the gradually increasing green color of the heart (Figure 3A) and the rest of the animal (Figures 3B–3F) during the procedure.

**Note:** The most critical aspect of the injection procedure is the quality of the microcapillary, which must have a sufficiently large tip diameter to prevent clogging, in particular when injecting the larger *C. reinhardtii* algae. If the microcapillary is clogged and an ejection of the algae suspension is impossible, the first option is to increase the pressure stepwise up to 2 bar and the pulse duration up to 200 ms.

### Visualization of microalgae and blood vessels in the brain

⌚ Timing: 30 min (step 12)

⌚ Timing: 16 h (step 13)

⌚ Timing: 3 h (step 14)

⌚ Timing: 2.5 h (step 15)

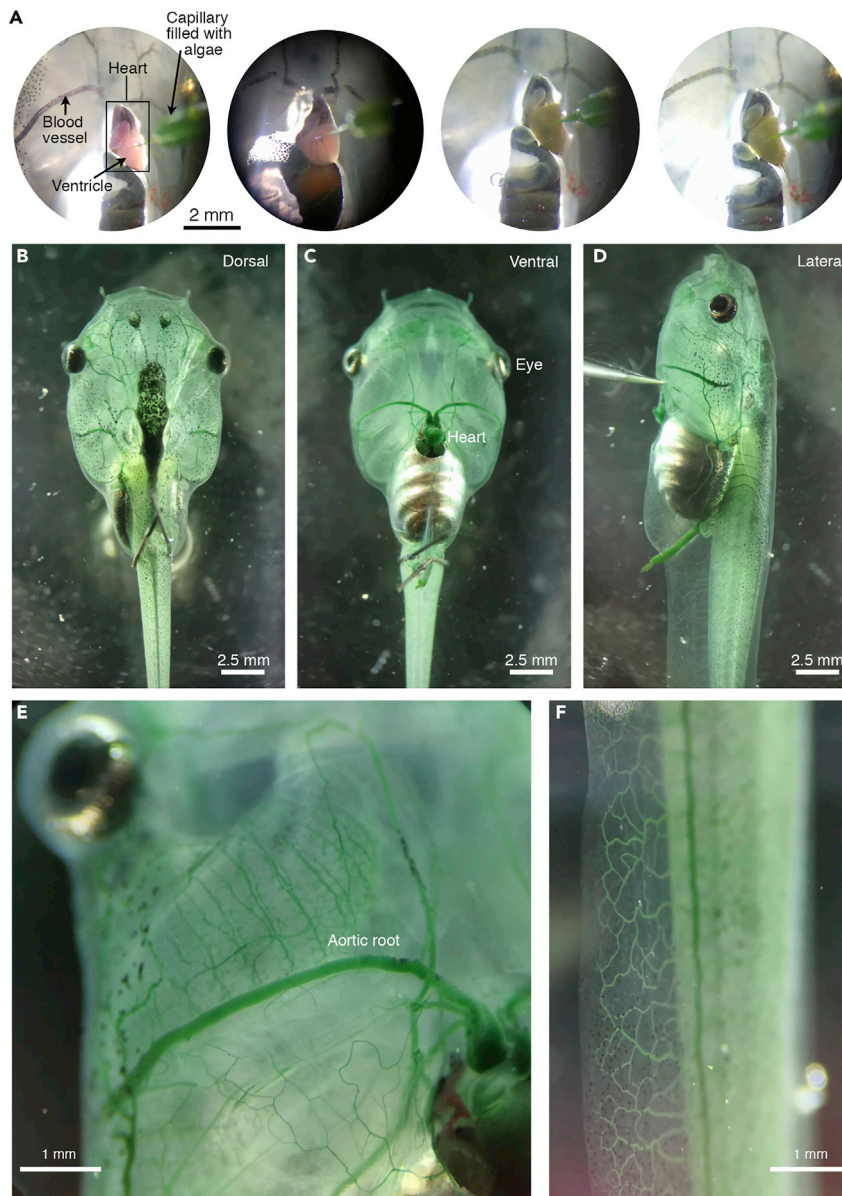
⌚ Timing: 15 min (step 16)

⌚ Timing: 1 h (step 17)

⌚ Timing: 45 min (step 18)

This major step describes the preparatory procedures for the anatomical visualization of the vascular system in the brain of *Xenopus laevis* tadpoles after the transcardial injection of the microalgae.

12. Euthanasia of the tadpoles and extraction of the brain for imaging the brain and vascular system.
  - a. Free the deeply anesthetized tadpole from the mechanical fixation.
  - b. Decapitate the animal with a small scissor at the level of the upper spinal cord (the level coincides with the external position of the developing hindlimbs).
  - c. Remove the lower jaws and visceral organs with the same scissor.
  - d. Pin the skull with the dorsal side up onto the Sylgard floor with minutiae (0.2 mm).
  - e. Remove the skin of the skull with fine forceps and a microscissor.
  - f. Open the skull at the rostral end and remove the cartilaginous tissue above the brain until the decapitated end of the head.
  - g. The brain is now entirely visible from the entrance of the olfactory tracts to the first spinal segments
  - h. Carefully cut all cranial and spinal nerves and remove the brain from the skull.
13. Fixation of the brain and preparation for confocal microscopy.
  - a. Fixate the brain/spinal cord in 20 mL of freshly prepared 4% PFA for 8 h.



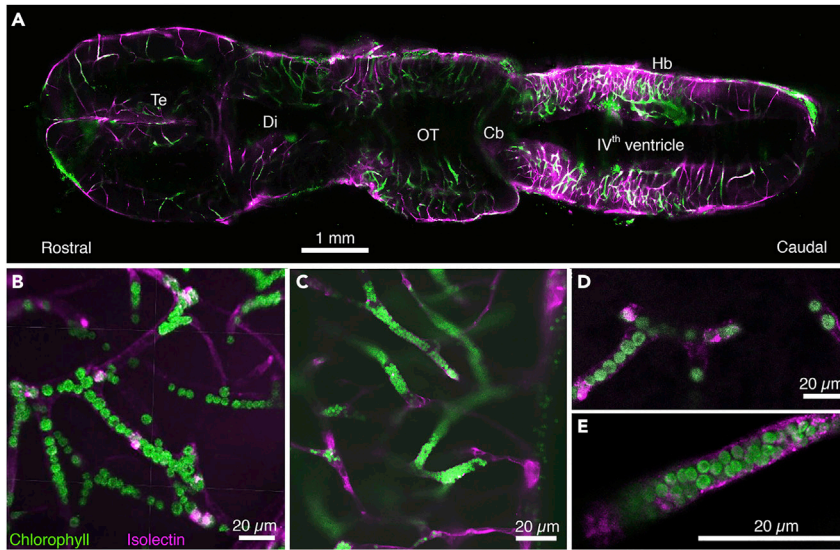
**Figure 3. Transcardial injection of algae suspensions completely fill blood vessels with phototrophic microorganisms**

(A) Ventral view of the exposed heart of a stage 53 *Xenopus* tadpole depicting the glass capillary injecting a *C. reinhardtii* suspension visualized by the gradual filling of blood vessels with the microorganisms over a period of 30 min, illustrated as four sequential images each separated by ~10 min (from left to right); note the progressively augmenting green colorization of the tissue.

(B–F) Overview (B–D) and higher magnification images (E and F) depicting a dorsal, ventral and lateral view of a stage 53 *Xenopus* tadpole after completion of the injection of cyanobacteria (*Synechocystis* 6803); note that the cyanobacteria have invaded the entire vascular system, likely filling even the smallest blood vessels in the body (E) and tail (F). Calibration bars in (B and E) apply to (C, D, and F), respectively.

Adapted from Özugur (2021) with permission under the CC BY-NC-ND license agreement.

- b. Wash the brain 3× in 0.1 M · L<sup>-1</sup> phosphate buffer.
- c. Transfer the brain into glycerol and leave it for 8 h for clearing.
- d. Mount the brain on depression slides with a coverslip above as preparation for confocal microscopy.



**Figure 4. Photosynthetic microorganisms in brain blood vessels**

(A) Confocal reconstruction of a *Xenopus laevis* whole brain illustrating isoelectin-labeled endothelial walls of blood vessels (magenta) and chlorophyll autofluorescence of transcardially injected *Synechocystis* 6803 (green).

(B–E) Confocal reconstruction of hindbrain tissue, depicting intravascular accumulations of *C. reinhardtii* (B and D) and *Synechocystis* 6803 (C and E); phototrophic microorganisms in green due to chlorophyll autofluorescence and isoelectin-stained endothelial walls of blood vessels in magenta.

Te, telencephalon; Di, diencephalon; OT, optic tectum; Cb, cerebellum; Hb, hindbrain. Adapted from Özugur et al. (2021) with permission under the CC BY-NC-ND license agreement.

#### 14. Imaging of whole mount brain preparations (Figure 4).

- a. Take the slide with the preparation (see above) and mount it onto the Mavig VivaScope RS-G4 for imaging.
- b. Use a long working distance objective that is adapted to the thickness of the preparation, especially to reach cellular resolution (e.g., 60× Olympus UPLSAPO60XO, NA 1.3, oil objective).
- c. Scan the brain tissue in optical sections of 0.5–1 μm thickness for the different wavelengths (Figures 4A–4E; Methods video S2). See troubleshooting 4.

**Note:** The chlorophyll of algae and cyanobacteria assign an autofluorescence to these microorganisms with an emission wavelength of 660 nm and 640 nm (chlorophyll a and b), respectively. To outline the vascular system in the brain, isoelectin conjugated with a compatible fluorochrome (Alexa Fluor™ 594 or Alexa Fluor™ 488) is most effective when injected along with the phototrophic microorganisms (see above). While a number of microscopes and objectives are suitable for the visualization, the description refers to a Mavig VivaScope RS-G4.

#### Measurement of oxygen and neural activity in semi-intact preparations

This major step describes the production of semi-intact preparations of *Xenopus* tadpoles that allow extended *in vitro* recordings of the oxygen dynamics and neuronal activity in the brain.

**Note:** Subsequent steps 15–21 describe the procedures required to monitor the photosynthetic oxygen production in viable semi-intact *Xenopus laevis* preparations. These steps are independent of and separate from steps 12–14 and have to be also performed immediately after the transcardial injection of the microalgae.

#### 15. Euthanasia of the tadpoles and production of semi-intact preparations.

- a. Free the deeply anesthetized tadpole from the mechanical fixation (after the injection of the microalgae).

- b. Decapitate the animal with a small scissor at the level of the upper spinal cord (the level coincides with the external position of the developing hindlimbs).
- c. Remove the lower jaws and visceral organs with the same scissor.
- d. Pin the skull with the dorsal side up onto the Sylgard floor with minutiae (0.2 mm).
- e. Remove the skin on the skull with fine forceps and a microscissor.
- f. Open the skull at the rostral end and remove the cartilaginous tissue above the brain until the decapitated end of the head.
- g. Leave all cranial and spinal nerves intact and the brain within the skull (in contrast to the outcome of step 12.h.).
- h. Identify and cut the ocular motor nerve that supplies the *superior rectus* eye muscle at its innervation site at the target muscle with a microscissor and free the nerve from connective tissue.
- i. Before the transfer into the recording chamber (Figures 5A and 5B), leave the preparation in the frog Ringer solution at 16°C–17°C in darkness for at least two hours to ensure a full recovery of the neuronal tissue from the dissection.

**Note:** The careful handling of the isolated preparation is key to the prolonged maintenance of functionality of the tissue for reliable measurements of O<sub>2</sub> concentration dynamics (see Özugur et al., 2020, 2021) and optimal recordings of neuronal activity at various sites within the brain or from cranial sensory or motor nerves (see e.g., Lambert et al., 2008; Gensberger et al., 2016).

### 16. Calibration of O<sub>2</sub>-sensitive electrodes.

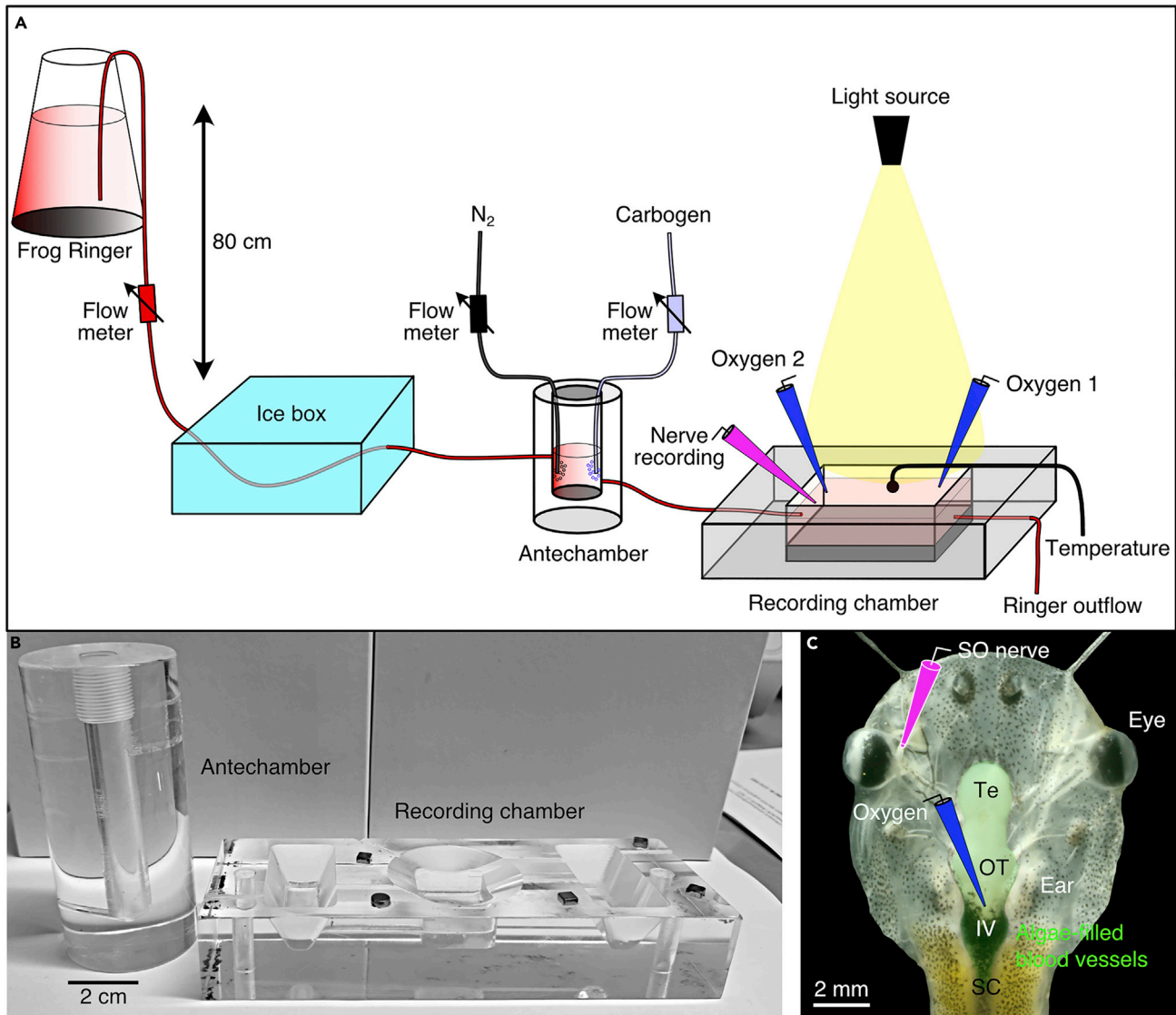
- a. Freshly calibrate the O<sub>2</sub> electrodes (Unisense A/S, Denmark) prior to each experiment using a solution with 0 μmol · L<sup>-1</sup> O<sub>2</sub> (0.1 M · L<sup>-1</sup> ascorbic acid in 0.1 M · L<sup>-1</sup> NaOH), 270 μmol · L<sup>-1</sup> O<sub>2</sub> (air saturated Ringer solution), and 1,350 μmol · L<sup>-1</sup> O<sub>2</sub> (carbogen saturated Ringer solution), at a temperature adjusted to 17°C. See user manual for more details (<https://unisense.com/products/o2-microsensor/>).

**Note:** This procedure is best performed with the software provided by the manufacturer of the O<sub>2</sub> electrodes and recording device (Unisense A/S, Denmark; <https://unisense.com/products/o2-microsensor/>).

### 17. Preparation of the recording chamber and installation of the recording devices.

- a. Mount the recording chamber (volume: ~2 mL; Figures 5A–5C) onto a metal support plate of ~50 × 40 cm (e.g., magnetic or with drill holes) underneath a binocular with a zoomable lens (2.5× to 20×).
- b. Place and fix the tip of a fiber thermometer in one of the corners of the recording chamber (Figure 5A).
- c. Connect the inflow of the recording chamber with a short thin tube to an antechamber (Figures 5A and 5B) dedicated to the increase or decrease of the O<sub>2</sub> level by aeration with carbogen or N<sub>2</sub>, respectively (Özugur et al., 2020).
- d. Connect the inflow of the antechamber with a 1-liter bottle of frog Ringer, placed at an elevated position of ~80 cm above the level of the recording chamber to provide a hydrostatic gradient for the constant supply of the recording chamber with frog Ringer solution.
- e. Lead the connecting tube through a flow meter and a box with crushed ice to cool the Ringer solution required to maintain a constant temperature in the recording chamber of 17 ± 0.2°C.
- f. Solidly mount a tripod with a 3-axes micromanipulator (see above) onto the metal base plate as support and positioning system for the electrophysiological recording electrode targeted at e.g., the *superior oblique* eye muscle nerve (Özugur et al., 2020, 2021).





**Figure 5. Recording of neuronal activity and O<sub>2</sub> production in semi-intact preparations of *Xenopus* tadpoles**

(A) Schematic depicting the individual components required to maintain viable semi-intact preparations in a recording chamber; frog Ringer solution is hydrostatically supplied from an elevated storage vessel through a tube system that allows an adjustment of the temperature (ice box) and the O<sub>2</sub> level (by aeration with N<sub>2</sub> or carbogen in an antechamber); recording electrodes for neuronal activity and O<sub>2</sub> are positioned with micromanipulators.

(B) Images of the antechamber and the recording chamber.

(C) Photograph, depicting a semi-intact head preparation and the placements of the electrodes for the recording of O<sub>2</sub> concentration dynamics in the IV<sup>th</sup> ventricle (blue electrode) and of multiunit spike discharge from the superior oblique motor nerve (pink electrode). OT, optic tectum; Te, telencephalon. (C), adapted from Özugur et al. (2021) with permission under the CC BY-NC-ND license agreement.

- g. Place the electrode holder, equipped with a connector for a polyethylene tube onto the micromanipulator.
- h. Attach a polyethylene tube (Inner diameter: 2 mm) to the connector and a 5 mL syringe to the other end.
- i. Connect the wire of the electrode holder to an amplifier (EXT 10-2F; npi electronics; Tamm, Germany) to allow extracellular recordings of multiunit activity (Lambert et al., 2008). See troubleshooting 5.

- j. Mount two O<sub>2</sub>-sensitive microsensors (Unisense A/S, Denmark) with tip diameters of 10 μm onto a piezo-stepper (Sensapex, Finland) attached to a micromanipulator (Sensapex, Finland) for fine, targeted advancements of the electrodes (Özugur et al., 2020).
- k. Connect the amplifier and the output of the O<sub>2</sub>-sensitive microsensors to an A/D board (CED 1401, Cambridge Electronic Design, UK). See [troubleshooting 6](#).
- l. Digitize (Spike 2, Cambridge Electronic Design, UK) O<sub>2</sub> concentration recordings and concurrent spike activity of the *superior oblique* motor nerve at 120 Hz and 5 kHz, respectively, and store the data on a computer for offline analysis.
- m. Mount a vertical bar with a clamp onto the metal base plate and fix a light source (Zeiss CL 6000 LED, with > 150 W, 600 lm) at a distance of 5 cm above the recording chamber for manually triggered illumination of the preparation; set the light intensity to 100 μE.

**Note:** The preparation of the recording chamber is only required once and can be left assembled and in place following excessive cleaning of the chambers and the tubing after each experiment with ddH<sub>2</sub>O. Also, the entire setup has to be placed inside a light-proof hood, cabinet or room to provide a dark environment.

18. Installation of the isolated preparation in the recording chamber and placement of the recording electrodes for O<sub>2</sub> and neuronal activity.
  - a. Ensure that the flow of the Ringer solution produces a temperature of 17°C inside the recording chamber and that the level of the frog Ringer is sufficiently high to accommodate the preparation with ~3 mm between the most dorsal anatomical structure and the Ringer surface.
  - b. Place and mechanically secure the isolated preparation in the center of the recording chamber with minutiae (0.2 mm).
  - c. Position one freshly calibrated O<sub>2</sub>-sensitive microsensor (see above) with the micromanipulator inside the IVth ventricle by targeting the small opening, naturally forming between the caudal end of the cerebellum and the rostral end of the choroid plexus to monitor the O<sub>2</sub> concentration in the brain.
  - d. Position the second freshly calibrated O<sub>2</sub>-sensitive microsensor (see above) with the micromanipulator next to the preparation in the recording chamber at a distance of ~2 mm at the same dorso-ventral level below the Ringer surface to monitor the O<sub>2</sub> concentration in the bath solution.
  - e. Pull a set of electrodes on a P-87 Brown/Flaming electrode puller from borosilicate glass (see above) and individually break the tip of the electrode to fit the diameter of the *superior oblique* motor nerve.
  - f. Place the tip of the recording electrode with the micromanipulator at the distal end of the transected *superior oblique* motor nerve (see above) and gently pull the nerve for a length of 1–2 mm into the electrode by providing negative pressure with the 5 mL syringe.
  - g. Visually ensure that the nerve tightly fills the tip of the electrode and remains mechanically stable within the electrode.
  - h. Move the electrode with the micromanipulator to avoid any stretching of the nerve.
  - i. Finalize the procedure by letting the preparation recover in darkness for 30 min.

**Note:** The success of the suction electrode recording will be immediately seen at the computer screen by the multiunit spike discharge illustrated by the software employed for digitization, data recording and storage (CED 1401, Spike2; see above). The firing rate of the multiunit discharge will be in the range of 30–70 impulses/s (Soupiadou et al., 2018). The software also allows monitoring the concurrent O<sub>2</sub> concentration inside the brain and the bath chamber in real-time. This represents the final step prior to the experiments that commence with the light-triggered increase of the O<sub>2</sub> concentration in the brain.

## EXPECTED OUTCOMES

This protocol describes the transcardial injection of *C. reinhardtii* or *Synechocystis* 6803, demonstrating a unique and highly efficient means to incorporate photosynthetic microorganisms into the brain of a vertebrate species. Microalgae, injected as highly concentrated suspensions, accumulate inside the vascular system of *Xenopus laevis* tadpoles (Figure 3) including blood vessels in the brain (Figure 4). The smaller prokaryotic cyanobacteria form tight cell packages in all blood vessels, while larger eukaryotic *C. reinhardtii* microalgae form pearl chain-like arrangements. Independent of the species, however, the transcardial injection of both types and the natural transport by the heart beat-driven blood flow into the vascular periphery produces a high microalgae density inside brain blood vessels. Comparison with the pattern of isolectin staining of the endothelial cells of the vessel walls confirmed that both species of microalgae invade virtually all blood vessels, likely including even the thinnest capillaries. The dense accumulation of phototrophic microorganisms inside the brain thus provides an excellent prerequisite for efficient photosynthetic O<sub>2</sub> production upon illumination of the mostly transparent tissue. To test the efficiency and suitability of this approach, the generation of viable semi-intact whole-head preparations of *Xenopus* larvae (Lambert et al., 2008) turned out to be a key element to establish this unique method. This experimental model was in fact crucial to demonstrate that phototrophic microalgae inside the brain of a vertebrate have the capacity to produce O<sub>2</sub> upon illumination and to rescue neuronal activity under hypoxic conditions.

## LIMITATIONS

Transcardial injection of microalgae might fail to reach the entire vascular system. In fact, an unknown fraction of the microalgae might permanently amass in the atrium of the heart and, thereby, become excluded from the circulation. Despite the apparent invasion of the entire vascular system of the brain by the microalgae, high-resolution confocal imaging studies will eventually have to visually confirm that all or at least the vast majority of blood vessels contain microorganisms. While the injected volume and species of microalgae obviously are able to produce measurable amounts of O<sub>2</sub>, the production rate might be enhanced by choosing smaller species or by a pharmacological pre-treatment of the vascular system yielding vessel dilation. The total O<sub>2</sub> production upon illumination might be underestimated, given its fast consumption by nerve and glia cells in the brain, once O<sub>2</sub> becomes available in the tissue. While larger amounts or longer periods of photosynthetic O<sub>2</sub> production might be desirable, it might also cause undesired oxidative stress. However, independent of these yet to explore details, this protocol represents a unique method and a first successful step in supplying the brain as well as potentially other organs with O<sub>2</sub>, produced by inserted phototrophic microorganisms.

## TROUBLESHOOTING

### Problem 1

Green algae or cyanobacteria fail to grow. ([before you begin](#) steps 1–4).

### Potential solution

Optimize microalgae growth by ensuring stable and physiologically appropriate light conditions and ambient temperature, by employing buffers with correct composition of ingredients and by preventing contamination with bacteria/yeast.

### Problem 2

Pressure application to the injection capillary does not cause microalgae ejection. ([step-by-step method details](#) step 11).

### Potential solution

Remove air bubbles from the capillary or fill a new capillary with the microalgae solution. During filling, avoid formation of air bubbles inside the capillary.

### Problem 3

Pressure application to the injection capillary fails to transcardially fill blood vessels with microalgae despite the absence of air bubbles in the capillary. ([step-by-step method details](#) step 11).

### Potential solution

Replace the injection capillary by another one with a larger tip diameter, because the tip of the capillary might have been too small and blocked by the microalgae solution.

### Problem 4

The brain tissue is opaque and unsuitable for confocal imaging. ([step-by-step method details](#) step 13).

### Potential solution

The clearing solution might have been contaminated or old. Redo all steps of the clearing protocol with fresh solution.

### Problem 5

No spike discharge can be monitored by the recording electrode despite the morpho-physiological integrity of the recorded cranial nerve. (See [step-by-step method details](#) step 17).

### Potential solution

The recording and/or the ground electrode in the bath chamber is not connected to the amplifier. Ensure the closure of the electrical circuits.

### Problem 6

O<sub>2</sub> monitoring shows extremely high, low, or drifting values. (See [step-by-step method details](#) step 17).

### Potential solution

Analyze potential errors in the calibration of the O<sub>2</sub> electrode such as an old (i.e., partially oxidized) ascorbate solution or temperature changes during bubbling of the calibration solution. Also, refer to the manual of the O<sub>2</sub> electrode for specific solutions (<https://unisense.com/products/o2-microsensor/>).

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Hans Straka ([straka@lmu.de](mailto:straka@lmu.de)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

This paper does not report original code. Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

## SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.xpro.2022.101250>.

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## AUTHOR CONTRIBUTIONS

Conceptualization, H.S., J.N., and L.K.; methodology, S.O., M.N.C., R.S., and H.S.; validation, S.O. and H.S.; formal analysis, S.O.; investigation, S.O., M.N.C., R.S., and H.S.; resources, H.S., J.N., and L.K.; data curation, S.O.; writing - original draft, S.O. and H.S.; writing - review & editing, S.O., H.S., L.K., and J.N.; visualization, S.O. and H.S.; supervision, H.S. and J.N.; project administration, H.S.; funding acquisition, H.S., L.K., and J.N.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

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