

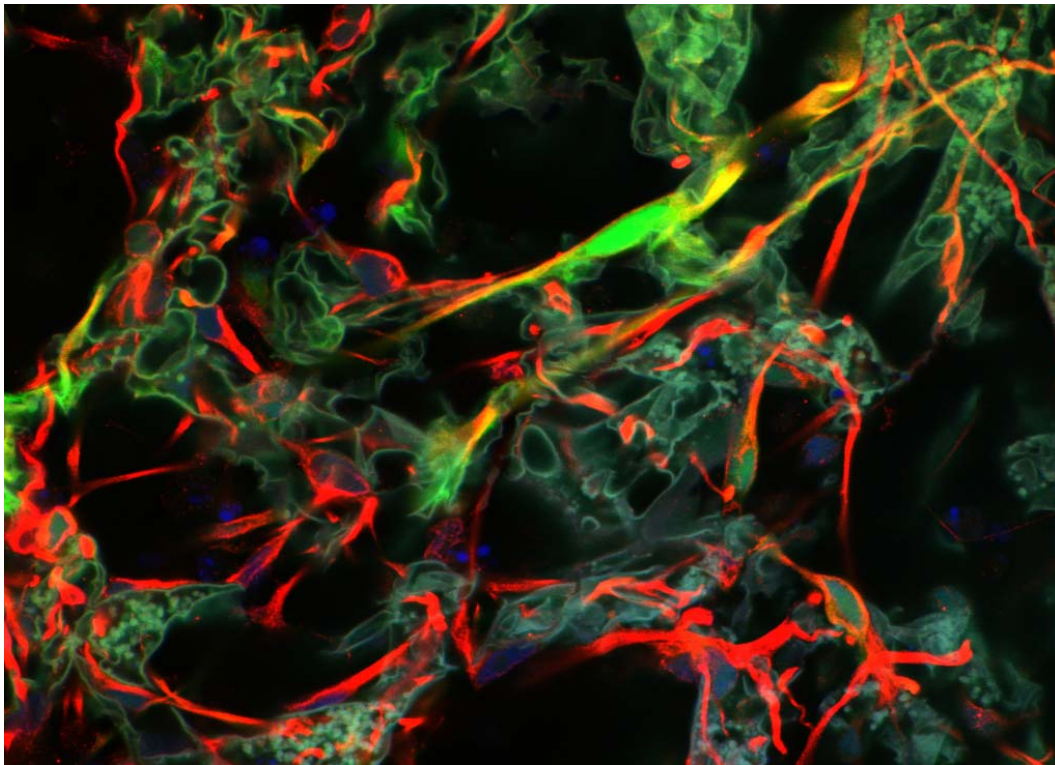
# 7<sup>th</sup> International Stem Cell School in Regenerative Medicine

*“Stem cells, biomaterials and nanotechnologies in regenerative medicine”*

## Practical Courses – Protocols

5 - 7 November 2009

Institute of Experimental Medicine, Academy of Sciences of the Czech Republic



# **The 7<sup>th</sup> International Stem Cell School in Regenerative Medicine “Stem cells, biomaterials and nanotechnologies in regenerative medicine” - Practical Course - Program**

**Venue:** Institute of Experimental Medicine (IEM), Academy of Science of the Czech Republic, v.v.i  
Videnska 1083  
14220 Prague 4,  
Czech Republic

**Responsible person:** Pavla Jendelova (PJ)

## **Group A) Biomaterials**

**Teachers:** Sarka Kubinova (SK), Katarina Masinova (KM), Vaclav Vanecek (VV), Dana Marekova (DM), Michal Doudera (MD), Pavlina Mackova (PM), Takashi Amemori (TA), Serhiy Forostyak (SF), Ales Hejcl (AH), David Arboleda (DA) Petr Lesny (PL)

## **Group B) Imaging - labeling cells in vitro with superparamagnetic nanoparticles, in vivo imaging of labeled cells using magnetic resonance imaging (MRI)**

**Teachers:** Mirka Kapcalova (MK), Nataliya Kozubenko (NK), Karolina Turnovcova (KT), Pavlina Mackova (PM), Vit Herynek (VH), Daniel Jirak (DJ), Martin Burian (MB), Takashi Amemori (TA), Petr Lesny (PL)

## **Group C) Patch-clamp recording and calcium imaging of stem cells**

**Teachers:** Miroslava Anderova (MA), Govindan Dayanithi (GD), Oksana Forostyak (OF), (PL), Iva Prajerova (IP), Jana Benesova (JB), Olena Butenko (OB), Pavel Honsa (PH), Helena Pavlikova (HP)

**Thursday, 5<sup>th</sup> November 2009**

## **Group A)**

**Venue:** Lecture hall, IEM 2<sup>nd</sup> floor; Laboratory of Cell Culture and Stem Cells, IEM 4<sup>th</sup> floor

8.30 – 9.00 Introduction, dividing into groups (PJ)

9.00 – 9.30 Introduction to cell culture techniques (VV)

**9.30 – 10.00 Coffee break**

10.00 – 12.30 Isolation of mesenchymal stem cells (MSCs) from the bone marrow (SK, KM)  
Preparation of biomaterials (hydrogels and nanofibers) for cell seeding (VV, MD)

- Dissecting the femurs and tibias from 4-week-old Wistar rats
- Extrusion of the bone marrow
- Plating the marrow cells
- Mounting nanofibers into scaffdex holders
- Shaping the hydrogels

12.30 – 13.00 Visit to GMP facilities for clinical grade cell production, IBC building (PL)

**13.00 – 14.00 Lunch**

14.00 – 15.00 Cell culture techniques (SK, KM, DM)

- Trypsinization of cells
- Counting cells using Bürker cell and Cellometer automatic cell counter
- Seeding the cells into 12 well plates with nanoparticles
- Comparing freshly seeded cells to cells labeled with nanoparticles after 3 days
- Fixation of labeled cells

**15.00 – 15.30 Coffee break**

15.30. – 16.00 Seeding cells on biomaterials and monitoring cell adhesion using a fluorescent microscope (SK, KM)

- Hydrogels
- Nanofibers

**Group B)**

**Venue:** Lecture hall, IEM 2<sup>nd</sup> floor; Laboratory of Cell Culture and Stem Cells, IEM 4<sup>th</sup> floor

8.30 – 9.00 Introduction, dividing into groups (PJ)

9.00 – 9.30 Introduction to cell culture techniques (VV)

**9.30 – 10.00 Coffee break**

10.00 – 12.30 Isolation of mesenchymal stem cells (MSCs) from the bone marrow (MK, NK)  
Preparation of phantoms for in vitro imaging (VH, PM)

- Dissection the femurs and tibias from 4-week-old Wistar rats
- Extrusion of the bone marrow
- Plating the marrow cells
- Preparation of gelatin
- Mixing the labeled cells with gelatin

12.30 – 13.00 Visit to GMP facilities for clinical grade cell production, IBC building (PL)

**13.00 – 14.00 Lunch**

14.00 – 15.00 Cell culture techniques (trypsinization, harvesting, cell counting in a Burger chamber and using a Cellometer counter) (SK, KM, DM)  
Cell labeling with nanoparticles (MK, NK)

**15.00 – 15.30 Coffee break**

15.30 – 17.00 Staining for iron (MK, PM)  
- Staining of labeled cell cultures and histological slices

**Group C)**

**Venue:** IEM Library, 2<sup>nd</sup> floor; Laboratory of Neurobiology, IEM 2<sup>nd</sup> floor

9.00 – 10.00 The basic principles of Ca<sup>2+</sup> imaging – GD  
- Role of Ca<sup>2+</sup> in physiology  
- Ca<sup>2+</sup>-sensitive dyes  
- Intracellular Ca<sup>2+</sup> measurements  
- Fast fluorescence microspectrofluorimetry  
- Single cell Ca<sup>2+</sup> measurements in real time  
- Characterisation of [Ca<sup>2+</sup>]<sub>i</sub> responses  
- Data acquisition and interpretation

**10.00 – 10.30 Coffee break**

10.30 – 11.30 The basic principles of patch clamp – MA  
- Preparation of cells or tissue slices for patch clamp recordings  
- Patch-clamp method  
- Whole-cell recording  
- Electrophysiological properties of cells  
- Cell labelling during patch clamp experiments and post-recording identification  
- Patch-clamp setup  
- Data acquisition – the software  
- Immunocytochemistry/immunohistochemistry

11.30 – 12.30 Visit to GMP facilities for clinical grade cell production, IBC building (PL)

**12.30 – 14.00 Lunch**

14.00 – 15.15 Experimental models of ischemic injury (PH)  
Focal cerebral ischemia - middle cerebral artery occlusion (MCAO)  
- Beginning the surgery  
- Transgenic EGFP/GFAP mice, anesthesia (atropine, pentobarbital)  
- MCAO  
- Triphenyltetrazolium chloride (TTC) staining of ischemic brain

**15.15 – 15.45 Coffee break**

- 15.45 – 17.00 Experimental models of ischemic injury (JB)  
Global cerebral ischemia – bilateral carotids occlusion (BCO)
- Beginning the surgery
  - Wistar rats, anesthesia (atropine, pentobarbital)
  - BCO

**Friday, 6<sup>th</sup> November 2009**

**Group A)**

**Venue:** Laboratory of Cell Culture and Stem Cells, IEM 4<sup>th</sup> floor

- 9.00-10.30 Measuring cell viability (SK, DM)
- WST-1 method

**10.30 – 11.00 Coffee break**

- 11.00 - 12.30 Immunohistochemical staining (SK, PM)
- Staining for cytoskeletal proteins (Phalloidin/actin)

**12.30 – 14.00 Lunch**

14.00 – 15.00 Fluorescent and confocal imaging (SK, VV)

**15.00 – 15.30 Coffee break**

15.30 – 16.30 Fluorescent imaging (SK, VV) (continued)

**Group B)**

**Venue:** Laboratory of Cell Culture and Stem Cells, IEM 4<sup>th</sup> floor

- 9.00 – 10.30 Experimental models of brain and spinal cord injury (KT, TA, DA)
- Cortical photochemical lesion
  - Balloon compression spinal cord lesion

**10.30 – 11.00 Coffee break**

11.00 - 12.30 Experimental models of brain and spinal cord injury, continued (KT, TA, DA)

**12.30 – 14.00 Lunch**

14.00 – 15.00 Cell transplantation into a photochemical lesion (KT, TA, MK)

**15.00 – 15.30 Coffee break**

15.30 – 16.30 Cell transplantation into a spinal cord compression lesion (KT, TA)

**Group C)**

**Venue:** Laboratory of Neurobiology, IEM, 2<sup>nd</sup> floor

9.00 – 10.30 Preparation of tissue slices for patch-clamp recordings (OB)

- Wistar rats – anesthesia (isofluran, pentobarbital)
- Transcardial perfusion with NMDG-solution
- Skull opening, isolation of the brain
- Slicing the tissue using a Vibrocot

**10.30 – 11.00 Coffee break**

11.00 – 12.30 Patch-clamp recordings (IP)

- Patch-clamp setup preparation
- Perfusion system
- Preparation of the recording pipette
- Visualizing the cells using a digital camera
- Approaching the cell with a pipette using micromanipulators
- Getting a seal, compensation, cell membrane rupture
- Recording in voltage-clamp/current-clamp mode
- Membrane properties of the recorded cell
- Fixation for post-recording identification

**12.30 – 14.00 Lunch**

14.00– 17.00 Immunocyto/histochemistry - confocal microscope (MA, JB, HP)

- Immunostaining of cells attached to a cover-slip or brain slices
- Frozen sections
- Confocal microscope

**19.30 – 22.30 Course Dinner at Restaurant Vikarka, Prague Castle**

**Saturday, 7<sup>th</sup> November**

**Group A)**

**Venue:** Laboratory of Cell Culture and Stem Cells, IEM 4<sup>th</sup> floor

9.30 – 10.00 Evaluation of isolated MSCs (MK)

10.00 – 11.00 Experimental models of spinal cord injury (balloon compression lesion) (TA, DA), implantation of seeded hydrogels (AH)

**11.00 – 11.30 Coffee break**

11.30 - 13.00 Experimental models of spinal cord injury (balloon compression lesion) (TA, DA), implantation of seeded hydrogels (AH), continued

**13.00 – 14.00 Lunch**

14.00 – 15.00 Histological evaluation (PJ)

15.00 – 16.00 Behavioral testing (DA)

- BBB
- Plantar test

16.00 Closing remarks

**Group B)**

**Venue:** Laboratory of Cell Culture and Stem Cells, IEM 4<sup>th</sup> floor; IKEM, Pavilion Z2

9.00 – 9.30 Evaluation of isolated MSCs (MK)

9.30 – 10.00 Walk to IKEM

10:00 – 10:15 Introduction to the MR facility, safety precaution (VH)

10:15 – 11:15 Measuring the relaxivities of labeled cell suspensions using a Bruker MiniSpec relaxometer at 0.5 T (VH, DJ, MB)

- sample preparation
- measurement sequences
- data processing

**11:15-11:30 Coffee Break**

11:30-13:00 Imaging and relaxation measurements of cell suspensions using a Bruker BioSpec spectrometer at 4.7 T (VH, DJ, MB)

- sample preparation
- sequence selection
- data processing

**13:00-14:00 Lunch**

14:00 -16:00 In vivo imaging of an experimental animal (rat) with labeled cells transplanted into a spinal cord lesion and/or an animal with cells transplanted into a brain with a photochemical lesion (VH, DJ, MB)

- animal preparation
- anesthesia
- animal handling in the spectrometer
- sequence selection
- measurement
- image processing
- closing remarks

### **Group C)**

**Venue:** IEM, Laboratory of Molecular Neurophysiology, 4<sup>th</sup> floor

9.00 – 10.30 Intracellular  $[Ca^{2+}]_i$  measurements in cultured cells (GD, OF)

- Cell cultures
- Dye loading onto cells
- Preparation of stimulus solutions (high  $K^+$ , ATP, glutamate and caffeine)
- Fast Fluorescence Photometry-setup-Instrumentation

### **10.30 – 11.00 Coffee break**

11.00 – 12.30 Intracellular calcium measurements (GD, OF)

- Real-time  $[Ca^{2+}]_i$  measurements on single cells
- Application of stimulus solutions
- Data collection and interpretation

### **12.30 – 14.00 Lunch**

14.00– 17.00 Intracellular  $[Ca^{2+}]_i$  measurements in supraoptic nucleus (SON) neurons in rats (GD, OF)

- Introduction to the physiology of SON neurons
- Isolation of SON neurons (Vasopressin and Oxytocin) from rats
- Enzymatic dissociation and dye loading
- Intracellular  $[Ca^{2+}]_i$  measurements in single neurons
- Application of physiological stimuli (high  $K^+$ , glutamate, ATP, caffeine)
- Observation of  $[Ca^{2+}]_i$  responses
- Data collection and interpretation



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## **The 7<sup>th</sup> International Stem Cell School in Regenerative Medicine “Stem cells, biomaterials and nanotechnologies in regenerative medicine” - Practical Courses - Protocols**

### **1. Cell culture techniques**

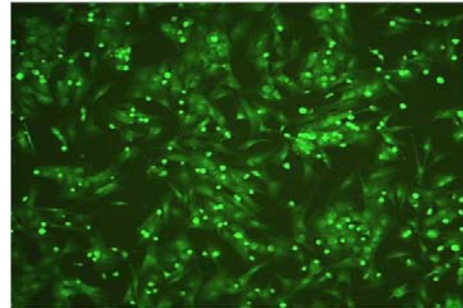
Rat mesenchymal stem cells (rMSCs) are pluripotent stem cells that are able to differentiate into multiple lineages and self renew. MSCs can be isolated from bone marrow and also from other mesenchymal tissues (for example, from adipose tissue or dental pulp). These cells play a crucial role in bone marrow stroma formation and regeneration. MSCs have the potential for therapeutic applications.

GFP rMSCs (fig) are MSCs isolated from rats expressing green fluorescent protein (GFP). Cells are cultivated as a monolayer in Dulbecco’s Modified Eagle’s Medium containing 10% fetal bovine serum and 2 µl/ml primocin in a humidified 5% CO<sub>2</sub> incubator at 37°C.

#### **1.1. Isolation of rMSCs**

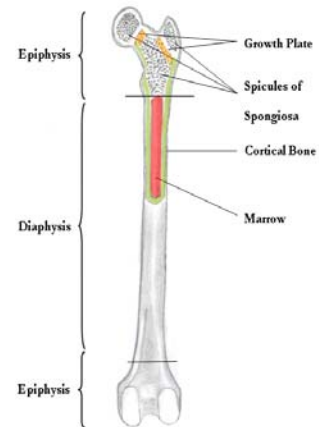
##### **1.1.1. Materials**

1. 4-week-old Wistar rats
2. 70% ethyl alcohol
3. Cotton wipes
4. Scissors, scalpel, tweezers
5. Petri dishes, pipettes
6. Needles, syringes
7. Racks, pipetter
8. Dulbecco’s Modified Eagle’s Medium (DMEM) – high glucose (4.5 g/l) with L-Glutamin (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS, PAA, Pasching, Austria) and with 0.2% primocin (Amixa, Gaithersburg, MD, USA). Other antibiotics can be used instead of primocin: 100 units/ml penicillin and 0.1 mg/ml streptomycin or gentamicin (50µl/ml).



### 1.1.2. Methods

1. Dissect the femurs and tibias from 4-week-old Wistar rats and clean them from the muscles with a scalpel in the operating room.
2. Put the bones in a Petri dish (PD), 1 animal per 1 PD, and transfer to the culture room.
3. Cut the ends of the bone and extrude the marrow with 10% DMEM using a needle and syringe.
4. Plate the marrow cells in the PD, top up with DMEM /10% FBS with 0.2 % primocin to 10 ml.
5. Place the PD with the cells in a humidified 5% CO<sub>2</sub> incubator at 37°C.
6. After 24 hours, the nonadherent cells can be removed by replacing the medium.
7. The medium will need to be replaced every 2-3 days as the cells grow to confluence.



## 1.2. Passaging and trypsinization of rMSCs

Efficient passaging may be achieved when the cultures are approximately 70 -80% confluent. The first passage after plating is usually taken as P0. Cells can be expanded up to passage 4 - 5 (P4 - P5).

### 1.2.1. Materials

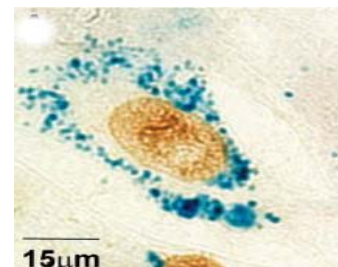
1. Cell culture plasticware: centrifuge tubes, pipettes, Petri dishes and tissue culture flasks, tips
2. Pipetter, automatic pipettes
3. Beaker for liquid waste, racks
4. Phosphate-buffered saline (PBS) pH 7.2
5. Trypsin solution (0.5%)
6. Fetal bovine serum (FBS)
7. Dulbecco's Modified Eagle's Medium (DMEM) – high glucose (4.5 g/l) with L-Glutamin (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS, PAA, Pasching, Austria) and with 0.2% primocin (Amaza, Gaithersburg, MD, USA). Other antibiotics can be used instead of primocin: 100 units/ml penicillin and 0.1 mg/ml streptomycin or gentamicin (50µl/ml).
8. Bürker cell or Cellometer automatic cell counter

### 1.2.2. Methods

1. Remove the culture medium by aspiration from the Petri dish (PD) or tissue culture flask (TCF).
2. Wash cells with 10 ml of PBS, remove the PBS by aspiration from the culture plasticware, repeat twice.
3. Add 1 ml of the dissociation reagent trypsin (0.5%), incubate cultures in a 37°C incubator 2 - 5 min until the cells detach. Observe the culture using a microscope.
4. Add 1 ml of FBS.
5. Add 6 ml of PBS, wash the PD or TCF and transfer the solution to a suitable centrifuge tube (12 ml), repeat twice.
6. Centrifuge at 1000 rpm, for 5 min at RT.
7. Prepare two new TCF, add 12.5 ml DMEM /10% FBS and label the TCF with the name of the cells, the date of isolation, the date of passaging and the passage number.
8. Discard the supernatant, add 1 ml of media, gently resuspend the pellet, divide the pellet in half and transfer each half to a new TCF.
9. Place the cultures in a humidified 5% CO<sub>2</sub> incubator at 37°C.  
or
10. Discard the supernatant and refill to 1 ml with PBS, repipette and transfer 10 µl of the suspension to a Bürker cell, count the cells.
11. Add the PBS to the centrifuge tube with the cell pellet and centrifuge at 1000 rpm, for 5 min at RT.
12. Discard the supernatant and place the cell suspension in a PD, TCF or other cell culture plasticware according to the number of cells.
13. Place the cultures in a humidified 5% CO<sub>2</sub> incubator at 37°C.  
or
14. Prepare the cell suspension for transplantation according to the number of cells.
15. Place on ice.

### 1.3. Cell labeling with magnetic nanoparticles

Non-invasive cellular imaging allows the real-time tracking of grafted cells as well as the monitoring of their migration. Visualizing the migration of transplanted cells *in vivo* is essential for pre-clinical studies in rodents and potentially also in humans.



For cellular MR imaging, cells need to be labeled with an MR contrast agent in order to visualize them in the host tissue. Contrast agents suitable for cell labeling must not only have high relaxation rates, but they also should not affect the cells' viability or their ability to differentiate and migrate towards the target tissue. In addition, the contrast agent must be either incorporated into the cell cytoplasm (intracellular cell labeling) or bound to the cell surface (extracellular cell labeling).

### 1.3.1. Materials

1. Adherent cell culture, 40 – 50% confluent
2. Solution of SPIO nanoparticles (e.g. Endorem, Guerbet Roissy, France). Stock solution contains 11.24 mg of iron per 1 ml of Endorem<sup>®</sup>. PLL-coated, D-mannose-coated and poly-dimethylacrylamide-coated iron oxide nanoparticles and uncoated  $\gamma$ -Fe<sub>2</sub>O<sub>3</sub> nanoparticles are used at a concentration of 4.4mg of iron per 1 ml of suspension.
3. Cell culture plasticware: 12 - 24 well plates, Petri dishes or tissue culture flasks, tips, centrifuge tubes, pipettes
4. Pipetter, automatic pipettes
5. Beaker for liquid waste, racks
6. Phosphate-buffered saline (PBS) pH 7.2
7. Trypsin solution (0.5%)
8. Fetal bovine serum (FBS)
9. Dulbecco's Modified Eagle's Medium (DMEM) – high glucose (4.5 g/l) with L-Glutamin (PAA, Pasching, Austria) supplemented with 10% fetal bovine serum (FBS, PAA, Pasching, Austria) and with 0.2% primocin (Amixa, Gaithersburg, MD, USA). Other antibiotics can be used instead of primocin: 100 units/ml penicillin and 0.1 mg/ml streptomycin or gentamicin (50 $\mu$ l/ml).
10. Bürker cell or Cellometer automatic cell counter

### 1.3.2. Method

1. Trypsinize the rMSCs or any adherent cells according to the trypsinization protocol.
2. Discard the supernatant and refill to 1 ml with PBS, same choices as on the previous page repipette and transfer 10  $\mu$ l of the suspension to a Bürker cell, count the cells.
3. Add the PBS to a centrifuge tube with cell pellet and centrifuge at 1000 rpm, for 5 min at RT.
4. Discard the supernatant and place the cell suspension in a PD, TCF or a 6 – 96 well plate according to the number of cells.

5. Add a SPIO nanoparticle suspension (10  $\mu$ L per 1 ml of culture medium, corresponding to a final concentration of 112.4  $\mu$ g of iron per 1 ml of culture medium) to a culture of rat MSCs or any adherent cells.
6. After 72 hours wash out the contrast agent.
7. Harvest the cells according to the trypsinization protocol and use them for either *in vitro* MR tracking or transplantation into animal models of disease or injury.

#### **1.4. Fixation of the cells**

##### **1.4.1. Materials**

1. Cells in culture wells or Petri dishes
2. 4% Paraformaldehyde (4% PFA)
3. Phosphate-buffered saline (PBS) pH 7.2
4. Pipet, automatic pipettes same curiosity
5. Pipetter, tips
6. Beaker for liquid waste

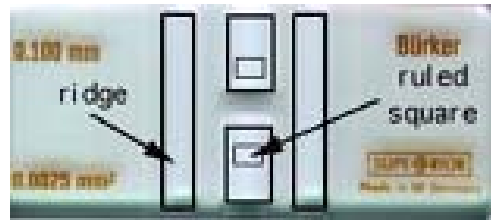
##### **1.4.2. Methods**

1. Remove the culture medium from the plasticware by aspiration.
2. Add PBS or distilled water and gently rinse the cells.
3. Remove the PBS from the plasticware by aspiration.
4. Add 4% PFA for 15 – 20 min at RT.
5. Wash cells 3x with PBS.
6. Add PBS and place in the refrigerator until staining for iron.

#### **1.5. Counting cells**

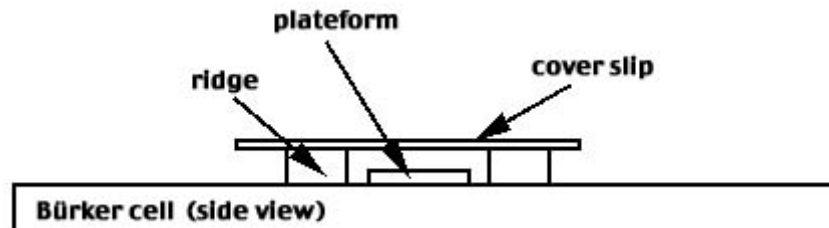
The number of cells in culture is important to know for subsequent experiments. It is possible to count cells in different ways. We use two approaches: 1. counting using a Bürker cell, 2. counting using a Cellometer.

### 1.5.1. Bürker cell

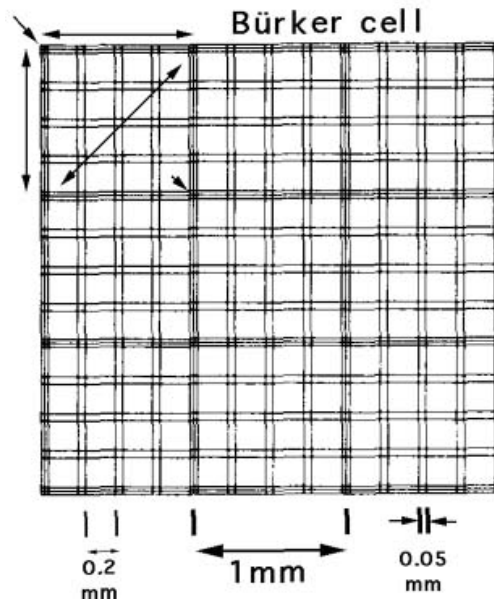


A Bürker cell consists of 2 identical ruled glass platforms mounted in a glass holder (see the drawing above). Each platform contains a ruled square measuring 3 by 3 mm (represented in the drawing below).

The surface of this square contains 9 smaller squares, each having a 1 square mm surface. On either side of the two ruled glass platforms is a raised ridge on top of which you place a cover slip.



The distance between the cover slip and the surface of the ruled area is 0.1 mm. The volume defined by a 1 mm square is thus 0.1 cubic mm



### 1.5.1.1. Counting cells using Bürker cell

- Mix the cell sample and pipette 20 µl into a microcentrifuge tube.
- Add 20 µl of trypan blue (0.5% w/v in PBS) and mix. This is a 1:1 dilution.
- Fix the cover slip.
- Add the cell suspension to the Bürker cell and load it by capillary action using a pipette.
- Allow the cells to settle.
- To avoid evaporation, count within one minute: the light of the microscope heats the sample.
- Count 25 small squares; for each square, in addition to the cells in the interior of the square, count the cells on 2 borders (upper and left) and on 2 corners (upper left and lower right).
- Total number of counted cells  $\times 10\,000 \times 2$  (dilution factor) = total cells/ml of sample

## **1.5. 2. Cellometer Auto T4 (Automatic cell counter)**

### 1.5. 2.1. Counting cells using automatic cell counter

- Mix the cell sample and pipette 20 µl into a microcentrifuge tube.
- Add 20 µl of trypan blue (0.5% w/v in PBS) and mix. This is a 1:1 dilution.
- Remove 20 µl and add to the counting chamber.
- Start the computer and the Cellometer.
- Open the program *Cellometer*.
- Place the counting chamber into the Cellometer.
- Click on *Display image* and focus the image using the focusing screw.
- Click on *Count*.
- Wait for the result.

## **1.6. Cell Proliferation Assay, (WST-1 method)**

Colorimetric assay (WST-1 based) for the nonradioactive quantification of cellular proliferation, viability, and cytotoxicity

### **1.6.1. Principle**

The stable tetrazolium salt WST-1 is cleaved to soluble formazan by a complex cellular mechanism that occurs primarily at the cell surface. This bioreduction is largely dependent on the glycolytic production of NAD(P)H in viable cells. Therefore, the amount of formazan dye formed directly correlates to the number of metabolically active cells in the culture.

Cells, grown in a 96-well tissue culture plate, are incubated with the ready-to-use WST-1 reagent for 0.5 - 4 hours. After this incubation period, the formazan dye formed is quantified with a scanning multi-well spectrophotometer (ELISA reader). The measured absorbance directly correlates to the number of viable cells.

## 1.6.2. Material

Adherent or suspended cells cultured in 96-well microplates.

There are several colorimetric assays that can analyze the number of viable cells; each is based on the cleavage of tetrazolium salts that are added to the culture medium. These assays do not require either washing or harvesting of the cells. The complete assay, from microculture to data analysis, can be performed in the same microplate.

### 1.6.2.1. WST-1 procedure

- Add 10  $\mu$ l/well Cell Proliferation Reagent WST-1 to cells already cultured in 100  $\mu$ l/well (1:10 final dilution) and incubate 2h at 37°C and 5% CO<sub>2</sub>.
- Measure the absorbance of the samples against a background control as a blank using a TECAN microplate reader. The wavelength for measuring the absorbance of the formazan product is 450 nm, the reference wavelength is 690 nm.

### 1.6.2.2. TECAN procedure

- Start computer
- Start TECAN Genios
- Open the program *Magellan* on the screen
- Start measurement →→
- Choose *WST universal*
- Write the name of the plate into the workspace
- Press button *Plate out*
- Insert plate
- Press button *Start*
- Results export to Excel: *File – Excel export* and save on USB flash drive

## 2. MR measurements

### 2.1. Preparation of phantoms containing suspensions of labelled cells for relaxometry measurements and MRI in vitro

To avoid the deposition of the cells at the bottom of the test vials, the cells should be suspended in 4% gelatin.

From a fixed cell suspension containing 2 million SPIO-labelled cells, several 1 mL samples can be prepared containing 200 000 – 2 000 000 cells/mL each. This range of concentrations ensures roughly 1 – 10 cells in one image voxel at a standard image geometry setting.

1. Concentrate (or dilute) 2 000 000 fixed cells in PBS to a volume of 0.5 mL. Mix the suspension well before each pipetting.
2. Place 250  $\mu$ L of the suspension (i.e., 1 000 000 cells) in a 0.5 mL Eppendorf test tube.

3. Place 125  $\mu\text{L}$  of the suspension (i.e., 500 000 cells) in a 0.5 mL Eppendorf test tube. Add PBS to 250  $\mu\text{L}$ .
4. Place 62.5  $\mu\text{L}$  of the suspension (i.e., 250 000 cells) in a 0.5 mL Eppendorf test tube. Add PBS to 250  $\mu\text{L}$ .
5. Place 31  $\mu\text{L}$  of the suspension (i.e., 125 000 cells) in a 0.5 mL Eppendorf test tube. Add PBS to 250  $\mu\text{L}$ .
6. Place 16  $\mu\text{L}$  of the suspension (i.e., 62 500 cells) in a 0.5 mL Eppendorf test tube. Add PBS to 250  $\mu\text{L}$ .
7. Prepare 8% gelatin from porcine skin in water. Suspend 0.8 g of gelatin in 10 mL of water and heat to 90°C.
8. Place 250  $\mu\text{L}$  of warm gelatin into each Eppendorf test tube. Mix well using a vortex and quickly cool on ice.

The same procedure should be repeated with cells labelled with different nanoparticles as well as with unlabelled cells that will serve as a control.

### **2.1.1. MR relaxometry at 0.5 T (Bruker MiniSpec)**

Gelatin cell samples should be measured at room temperature, otherwise the gelatin partially melts and the samples become inhomogeneous.

Put the test tube with the gelatin cell sample into the MR cuvette and place it into the probe of the relaxometer. For T2 measurements, use a standard CPMG sequence (echos spacing TE=2 ms, repetition time TR= 5 s, number of acquisitions AC=16).

### **2.1.2. Magnetic resonance imaging of cell phantoms at 4.7 T (Bruker BioSpec).**

Put a set of 10 gelatin cell samples into a holder. Place the holder into the resonator coil in the magnet bore of the spectrometer.

For *in vitro* measurements use standard imaging sequences that are used for *in vivo* measurements:

1. Localizer (fast low angle gradient echo sequence) used for positioning of the sample.
2. T2-weighted turbospin echo sequence with the following parameters: TR = 2000 ms, effective echo time TE = 42.5 ms, turbo factor = 4, number of acquisitions AC = 16, matrix 256x256, slice thickness 0.5-1 mm. The field of view is chosen according to the size of the samples.
3. T2\*-weighted gradient echo sequence with the same geometry and the following parameters: TR=80 ms, TE=5 ms, AC=32.
4. T2 relaxation time measurement using CPMG sequence, parameters: echos spacing TE = 8.6 ms, AC = 1, TR=5000 ms, matrix 128x128, slice thickness 2 mm. The field of view is chosen according to the size of the samples.

## **2.2. In vivo MRI**

Superparamagnetic particles have a substantial impact on both T2 and T2\* relaxation times. Therefore, both T2 (spin echo, turbospin echo) and T2\*-weighted (gradient echo) sequences can

be used. An implant with superparamagnetic particles will manifest itself as a hypointense area. Although T2\*-weighted gradient echo is more sensitive to the presence of superparamagnetic particles, we strongly prefer a T2-weighted turbospin echo sequence, as it provides better anatomical images.

### **2.2.1. Animal preparation**

The animal should be anesthetized throughout the entire measurement. Simple MRI is relatively short, therefore intubation of the animal is not necessary and passive inhalation of the anaesthetic is sufficient for maintaining anaesthesia.

Place and fix the anesthetized animal into a heated MR-compatible holder. An airflow of 200-300 mL per minute is maintained through a mouthpiece. The concentration of the inhalation anaesthetic should be 1-2 % depending on the breathing frequency of the animal. The breathing frequency is maintained at approx. 1 breath per second. Monitor the animal's breathing throughout the entire experiment. A dedicated surface head coil is placed and fixed over the rat's head.

In the case of an animal with a spinal cord lesion, fix the animal on its back into a holder with an integrated spine RF coil.

### **2.2.2. Magnetic resonance imaging**

Place the holder with the animal into the magnet bore.

Connect and tune the RF coil.

An automated procedure for shimming, setting of transmitter power, frequency and receiver gain should be sufficient for MR imaging.

Sequences employed:

1. Localizer (fast low angle gradient echo sequence). Reposition the animal, if necessary.
2. T2-weighted turbospin echo sequence with the following parameters: TR = 2000 ms, effective echo time TE = 42.5 ms, turbo factor = 4, number of acquisitions AC = 16, matrix 256x256, slice thickness 0.5-1 mm. The field of view is chosen according to the size of the rat's head.
3. Measure the images in different orientations, if necessary (i.e., axial, sagittal, coronal directions).
4. T2\*-weighted gradient echo sequence with the same geometry and the following parameters: TR=80 ms, TE=5 ms, AC=32.

In the case of a spinal cord lesion, modify the geometry of the sequences according to the required field of view.

## **2.3. Staining for iron (Prussian Blue staining)**

### **2.3.1. Materials**

7. Fixed cells
8. Hydrochloric acid (0.5% HCl)

9. One gram of potassium ferrocyanide ( $K_4[Fe(CN)_6].3H_2O$ ; Sigma, St. Louis, USA) dissolved in 100 ml of 0.5% HCl
10. 70% ethyl alcohol (EtOH)
11. Aluminium sulfate (5%  $Al_2(SO_4)_3$ ; Sigma, St. Louis, USA)
12. Nuclear fast red (Sigma, St. Louis, USA) diluted in 5% aluminium sulfate

### **2.3.2. Method**

1. Remove the PBS or distilled water from the culture plasticware by aspiration.
2. Wash in alcohol (70%) for 2 minutes.
3. Add PBS or distilled water and gently rinse the cells.
4. Add potassium ferrocyanide ( $K_4[Fe(CN)_6].3H_2O$ ) for 30 min to produce ferric ferrocyanide (Prussian blue).
5. Counterstain the cell nuclei with nuclear fast red or hematoxylin.

## **3. Biomaterials**

### **3.1. Preparation of nanofibers for cell culture**

Do steps 2-4 in a laminar box

1. Remove the nanofibrous textiles from the support substrate, cut into squares (1.5 x 1.5 cm), sterilize from both sides by UV light and fix into CellCrown<sup>TM</sup> 24 inserts (Scaffdex, Tampere, Finland).
2. To avoid cytotoxicity of the nanofibers due to residual monomers and organic solvents, wash out the nanofibers in distilled water before cell seeding. Place the inserts with nanofibers into 200 ml of sterile distilled water in glass bottles, and change the water five times under sterile conditions in a laminar box.
3. Prior to cell seeding, place the inserts with nanofibers into 24-well plates, wash twice with culture medium and put into an incubator for 0.5 h with 0.5 ml of the culture medium in each well.
4. Prepare a cell suspension and add  $2 \times 10^4$  cells per well/nanofiber. Also add the cell suspension into 2-3 wells without nanofibers to compare cell growth. Put the plate into the incubator.

### **3.2. Measurement of the viability of cells seeded on nanofibers**

Do all steps in a laminar box

1. To avoid including those cells that escaped from the inserts and grew on the bottom of the culture wells in the viability assays, place the inserts with seeded nanofibers into new wells with 0.5 ml fresh culture medium before viability measurements.
2. Add WST-1 reagent (10  $\mu$ l/100  $\mu$ l of the medium) into each well, and let the plates incubate for 1-3 h at 37°C to form formazan. Also add the WST-1 reagent into a well containing only culture medium to measure background absorbance.
3. Transfer 100 or 200  $\mu$ l of formazan-containing medium from each well into a 96-well plate. Measure the absorbance using an ELISA plate reader at a wavelength of 450 nm against a reference wavelength of 690 nm. Subtract the background absorbance of the medium from the viability values.

### **3.3. Immunofluorescence staining for F-actin and DAPI**

Place the nanofibers, fixed in CellCrown inserts, into culture wells during the staining process. Use 0.5ml of solution per well during the staining process.

1. Wash the cells three times with 0.1 M PBS, fix the cells in 4% paraformaldehyde (in 0.1 M PBS, pH 7.4) for 15 min, and wash them again three times with PBS.
2. Add 0.1 M PBS with Triton X-100 (0.5%, Sigma, St. Louis, MO, USA) for 10 min, repeat three times.
3. To label F-actin, incubate the cells with Alexa-Fluor 568 Phalloidin (Molecular Probes, Invitrogen, Paisley, UK) diluted 1:100 in 0.1 M PBS containing Triton X-100 (0.5%, Sigma) for 1.5h at room temperature.
4. Add PBS for 10 min.
5. To label cell nuclei, add 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) diluted 1:500 in 0.1 M PBS for 1 min. Wear gloves.
6. Add 0.1 M PBS for 10 min, repeat three times.
7. Remove the inserts from the wells, loosen the rings, remove the nanofibers and place the nanofiber pieces cell-side up”
8. Mount the nanofibers on glass slides with the use of Aqua Poly/mount (Polysciences, PA, USA)

## **4. Models of brain and spinal cord injury**

### **4.1. Permanent Middle Cerebral Artery Occlusion (MCAO) in the mouse**

#### **4.1.1. Materials and Methods**

Transgenic mouse (18-40 g, minimum 30 days old)

1% pentobarbital in 0,9% NaCl, 1 ml injection

Antiseptic solution

2 small forceps

1 small pair of scissors

Heating pad (36,5°C), microscope, source of light

Braided silk– USP 7/0

Silon monofil – USP 6/0

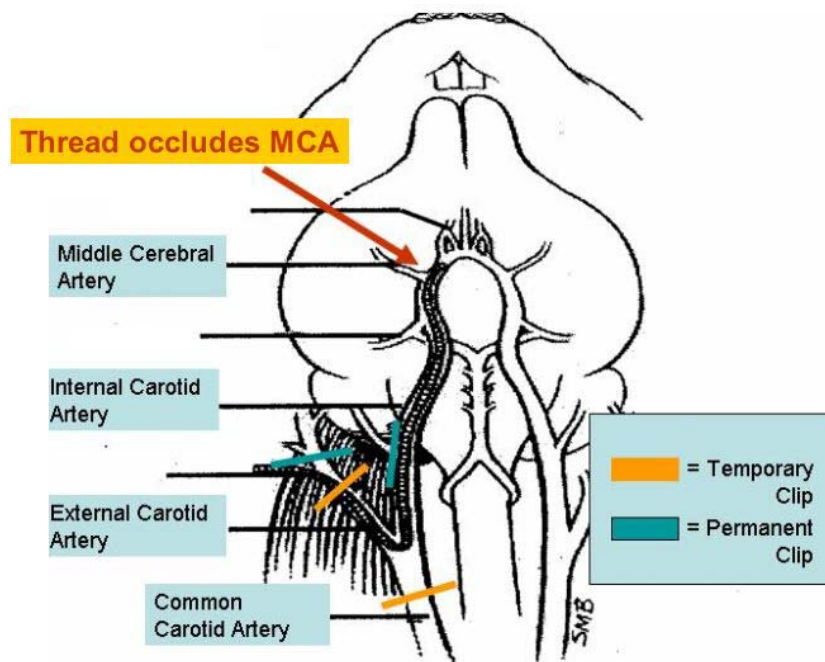
2 small clamps

Thread from silon monofil – USP 5/0 (150 um), 15 mm long, with widening on one side (220-240 um)

- 1) Anesthetize the animal – (animal weight in g x 0,9) x 10 = volume of 1% pentobarbital intraperitoneal (ul).
- 2) Fix the animal to a heating pad with tape and disinfect the skin on the neck.
- 3) Open the skin on the right side on the neck with small scissors in the rostro-caudal direction (cca 15 mm long).
- 4) Preparation of the common carotid artery and bifurcation of the internal carotid artery and external carotid artery.
- 5) Permanent ligation of the external carotid artery with braided silk – as rostrally as possible
- 6) Transient occlusion of the common carotid artery with a clamp – as caudally as possible
- 7) Temporary occlusion of the internal carotid artery with a clamp – as dorsally as possible.
- 8) Using scissors, cut a small hole into the external carotid artery and insert the filament.
- 9) Remove the clamp from the internal carotid artery.
- 10) Place a thread into the internal carotid artery through the common carotid artery, 9-10 mm from the bifurcation.
- 11) Fix the thread in the internal carotid artery with silk and cut it.
- 12) Remove the clamp from the common carotid artery. Suture the skin.

13) Inject c. 400 ul of sterile saline subcutaneously.

14) Leave the animal on the heating pad to recover from the anesthesia.

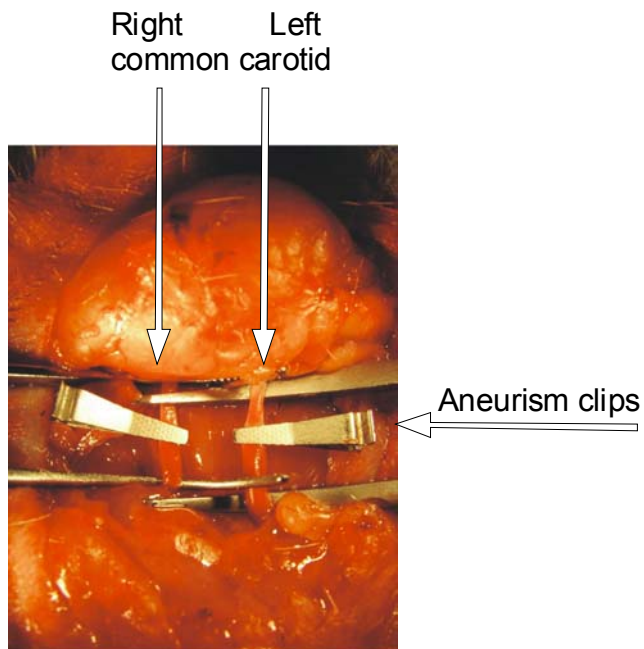


#### 4.2. Global cerebral ischemia

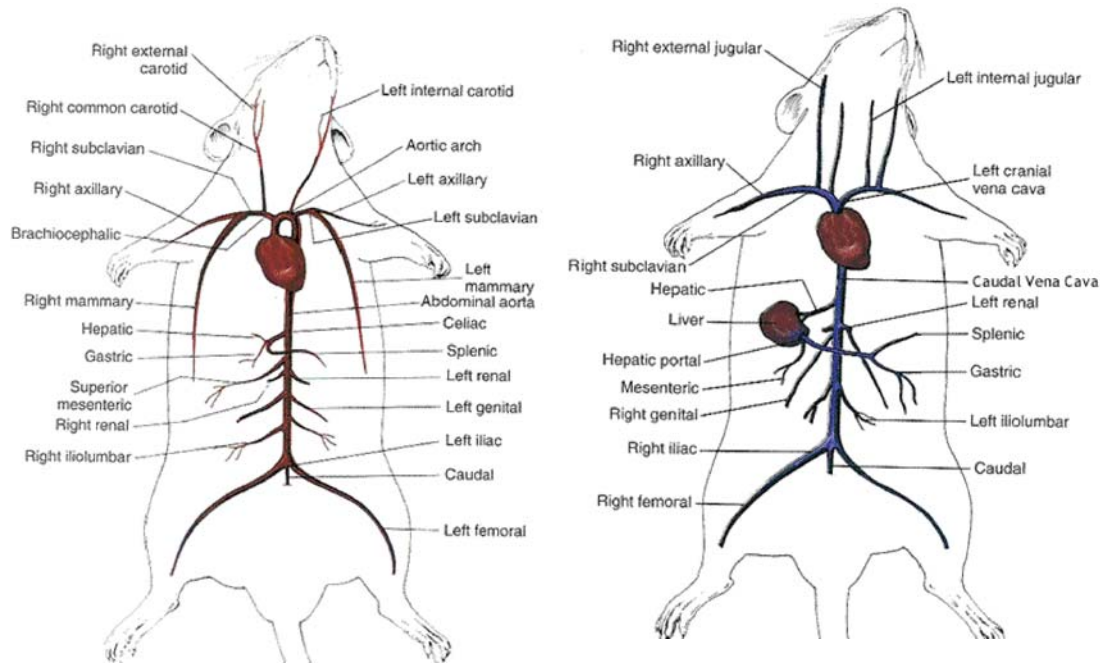
Male Wistar rat (7-9 weeks old, 220-260g) will be used for induction of global cerebral ischemia

1. Anesthetize the animal via an intraperitoneal injection of **sodium pentobarbital**. (65 mg/kg, i.e. 1.6 ml for a 250g rat).
2. Intubate the animal with a cannula tube and connect to a mechanical ventilator. Start **ventilation** with 33.3% O<sub>2</sub> and 66.6% N<sub>2</sub>.
3. Place the animal on a heating pad to hold the body temperature at 37°C.
4. Shave the neck area and disinfect the skin.
5. Superficially cut the skin in the middle of the throat with scissors.
6. Using tiny forceps, expose the common carotid arteries by blunt dissection.

7. Use aneurism clips to clamp both arteries.
8. Begin a 15-minute common **carotid artery occlusion**.
9. During occlusion ventilate the animal with **6% O<sub>2</sub> and 94% N<sub>2</sub>**.
10. Remove the clips and change the ventilation back to 33.3% O<sub>2</sub> and 66.6% N<sub>2</sub>.
11. Continue ventilation for 1 hour.
12. Suture the skin.
13. Inject cca 2 ml of saline subcutaneously to prevent dehydration.
14. Keep the animal on the heating pad until it awakes from the anesthesia.



**Fig. 1: Common carotid artery occlusion**



**Fig. 2: Rat circulatory system : arteries (left) veins (right)**

### **4.3. Model of an ischaemic cortical lesion – a photochemical lesion of the rat brain**

#### **4.3.1. Material / Reagents**

Stereotaxic apparatus for small rodents  
 Cold white light source  
 Surgical instruments (scissors, scalpel, forceps, surgical sutures)  
 Syringe  
 Rose Bengal sodium salt  
 0.9% NaCl  
 Disinfectant  
 Cotton wipes, cotton pads  
 Light-proof foil with a lesion-sized opening

#### **4.3.2. Anesthesia**

2% isoflurane or parenteral anesthesia (pentobarbital 40mg/kg IP body weight)  
 Wait for full and deep anesthesia (no response to pinching the tail or the paw!)

#### **4.3.3. Postoperative anesthesia/analgesia**

Carprofen 4-5 mg/kg SC or butorphanol 0.05mg-2mg/kg body weight SC

#### **4.3.4. Surgical procedure**

Mix the fresh rose bengal (0.16 g/ml 0.9% NaCl).  
 Prepare a syringe with the rose bengal solution (50  $\mu$ l/ 1 kg body weight); keep in the dark.  
 Shave the scalp and disinfect.

Make an incision on the skull, and remove skin and membranes from the skull surface; wipe the surface until dry.

Mark the desired position on the skull with a pencil.

Inject the rose bengal solution intravenously (use the tail vein or femoral vein); make sure the rat's auricles and nose become pink-violet.

Fix the rat in the stereotactic apparatus using rat ear bars.

Cover the rat's head with the foil so that you can see the marked position in the opening.

Set the light source just above the opening and allow the light source to shine on the skull for 8 minutes

Remove the foil and moisturize the skull using 0.9% NaCl.

Remove the animal from the stereotaxic apparatus.

Close the skin incision.

Administer analgesics.

Keep the animal under a warming light or on a heating pad until it begins to move.

Enable free access to food and drink.

Check the animal's status every day.

#### **4.4. Spinal cord injury (SCI) – Balloon induced spinal cord compression lesion**

Balloon compression creates an incomplete SCI and is used as an animal model of human SCI.

##### **4.4.1. Materials**

- 2-french Fogarty catheter (filled with saline or distilled water)
- Hamilton syringe (50  $\mu$ L), microinjector
- Scissors, dissecting forceps, scalpel handle and blade, curved hemostatic forceps, bone rongeurs
- Needle holder, suture materials
- Heating pad, thermometer

##### **4.4.2. Anesthesia**

3% isoflurane in air at a flow rate of 0.3 L/min

##### **4.4.3. Pre- and post-surgical treatment**

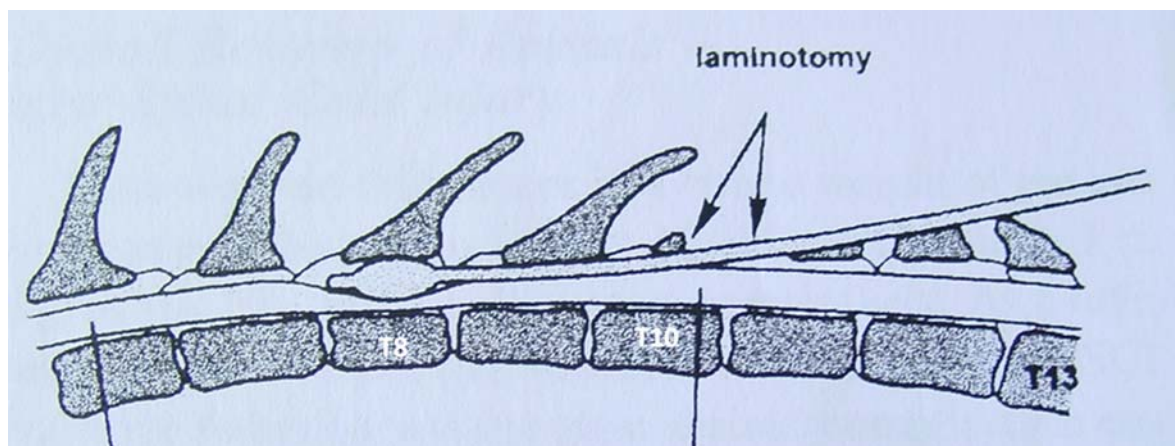
Gentamicin sulfate (5 mg/kg)

##### **4.4.4. Procedure**

1. After placing the rat into a closed chamber, administer isoflurane anesthesia until a surgical level of anesthesia is reached, then place the rat on a heating pad to maintain its

body temperature at around 37 °C; maintain this level of anesthesia using a mask covering the mouth and nostrils.

2. All hair from the incision site (above the thoracic and lumbar vertebrae) should be removed by scissors or an electric shaver.
3. The surgical area is cleansed with 70 % ethyl alcohol.
4. An incision of the skin is made by a single stroke of the scalpel between the thoracic vertebrae 5 and 13.
5. Bleeding from the cut should be stopped by pressing a piece of tissue paper on it.
6. The muscles and connective tissues are carefully separated from the vertebrae so that each spinal process of the vertebrae is clearly visible.
7. The animal's body is lifted by holding the spinal process of thoracic vertebra 8 with curved hemostatic forceps.
8. Under a dissection microscope, the spinal processes of T10 and T11 are removed by bone rongeurs, and the rongeurs are carefully introduced into the intervertebral foramen (between T10 and T11) to remove bone from the base of the spinal process (T10) in order to expose the spinal cord. Great care should be taken to avoid damaging the venous structures and the dura mater.
9. A balloon catheter is inserted in the opening of the vertebra (T10), then advanced forward so that the center of the balloon can reach T8 (about 1 cm rostral to T10).
10. After the catheter is settled at T8, the animal is released from the forceps holding its body and placed on a heating pad. The opening of the vertebra should be covered with saline.
11. Measure the body temperature to confirm that it is maintained at around 37°C.
12. The balloon is inflated with 15  $\mu$ L of saline or distilled water.
13. After 5 minutes, the balloon is deflated and removed.
14. The muscle and the skin are sutured, and the surgical area is cleaned with ethyl alcohol.
15. The animal is taken off anesthesia.
16. Care should be taken to assist in feeding and urination until the animals can perform these activities themselves.



Modified from Vanicky et al., J. Neurotraum 12:1399-1407, 2001

## **4.5. Acute spinal cord injury (hemisection) and the implantation of biomaterials (gels/nanofibers)**

### **4.4.1. Materials**

- Scalpel handle with blade, scissors, forceps, bone rongeur, 2 micro-pincettes, micro-needle holder, micro-scissors, self-retaining retractor
- Suture materials (Dafilon 10/0)
- Heating pad
- Hydrogel/nanofiber
- Dissecting microscope

### **4.5.2. Anesthesia**

- Initial narcosis: 5% isoflurane for 5 min., 0.5 L/min
- Pentobarbital 60 mg/kg, intraperitoneal injection
- Local anesthesia: Mesocaine, 2 ml

### **4.5.3. Pre- and postsurgical treatment**

- Gentamicin sulfate 5 mg/kg, i.m. for 3 days (to prevent bacterial infection)
- Atropine 1:5, 0.3 ml (0.04 mg/kg), i.m., single injection (to decrease parasympathetic innervation during surgery)
- After surgery, the animals are housed separately and are monitored until they awake from the anesthesia

### **4.5.4. Hemisection with gel /nanofiber implantation**

1. After a deep level of anesthesia is achieved, the animal's limbs are fixed to the surgical table with a lace. The fur at the site of implantation is removed with scissors, and the skin is cleaned with 70 % ethyl alcohol.
2. A 3 cm skin incision above the thoracic vertebrae is made with a single stroke of the scalpel (Th 6-13).
3. By separating the musculature from the vertebrae, the spinal processes become visible. The edges of the wound are held with a self-retaining retractor.
4. With the help of forceps, the animal is lifted to give the vertebral column an arched shape. Under the surgical microscope a laminectomy at the Th 8-9 level is carefully performed using a bone rongeur, and the spinal cord is exposed. The dura mater and the underlying vessels should not be damaged during exposure. The dura mater is cut longitudinally in the middle, and a hemisection on the left/right side is made with micro-

pincettes. The size of the removed neural tissue is approximately 2x2x2 mm, and bleeding is controlled with small pieces of absorbent cotton.

5. The same size of scaffold is placed into the dry cavity, trying to avoid bubble formation between the neural tissue and the biomaterial.
6. The dura mater is sutured with Dafilon 10/0 thread to keep the graft inside the cavity.
7. The muscles and skin are sutured, and the surgical area is cleaned with 70% alcohol.

## **5. Cell transplantation**

### **5.1. Transplantation of (human mesenchymal stem) cells into the rat brain**

#### **5.1.1. Material / Reagents**

Stereotaxic apparatus for small rodents  
Surgical instruments (scissors, forceps, scalpel, surgical sutures)  
Bur (ball-shaped, 1.6 mm diameter)  
Hamilton syringe (5  $\mu$ l with a conical needle)  
(Mesenchymal stem) cell suspension  
Bone wax  
Cotton wipes, cotton pads  
0.9% NaCl  
Skin disinfectant  
Immunosuppressive drugs (recommended)

#### **5.1.2. Anesthesia**

2% isoflurane or parenteral anesthesia (pentobarbital 40mg/kg IP body weight)  
Wait for full and deep anesthesia (no response to pinching the tail or the paw!)

#### **5.1.3. Postoperative anesthesia/analgesia**

Carprofen 4-5 mg/kg SC or butorphanol 0.05mg-2mg/kg body weight SC

#### **5.1.4. Surgical procedure**

Shave the scalp, disinfect the skin.  
Make a skin incision on the skull; remove the skin and membranes from the skull surface.  
Fix the animal in the stereotaxic apparatus using rat ear bars.  
Mark the desired position with a pencil.  
Carefully bur a hole at the marked point; remove the bone fragments using a wet cotton applicator.  
Remove the last layer of the bone using forceps; AVOID damaging the dura mater!  
Prepare the Hamilton syringe with the appropriate volume of the cell suspension; AVOID air bubbles!  
Fix the Hamilton syringe in the stereotaxic apparatus; check the suspension flow in the syringe.  
Set the syringe in the hole and lower the needle tip until it touches the dura mater.  
Slowly lower the needle to the desired position in the brain tissue.  
Wait for a minute to allow the brain to recover its normal shape

Inject the desired volume at a constant flow rate.  
Wait at least 5 minutes to allow the tissue and cells to adapt.  
Slowly remove the needle, then remove the animal from the stereotaxic apparatus.  
Use bone wax to treat the bone wound.  
Close the skin incision.  
Administer analgesics.  
Keep the animal under a warming light or on a heating pad until it begins to move.  
Enable free access to food and drink.  
Check the animal's status every day.

## **5.2. Cell transplantation into SCI**

### **5.2.1. Materials**

- Stereotaxic apparatus
- Hamilton syringe (50  $\mu$ L), Nano injector, glass pipette, saline
- Scissors, dissecting forceps, curved hemostatic forceps, bone rongeurs
- Needle holder, suture materials

### **5.2.2. Anesthesia**

2.5 % isoflurane in air at a flow rate of 0.3 L/min

### **5.2.3. Pre- and post-surgical treatment**

Cyclosporine (10 mg/kg) and ampicillin (50-100 mg/kg)

### **5.2.4. Procedure**

1. One week after SCI, the animal is anesthetized with isoflurane, and the operated area is reopened by scissors after removing hair and cleansing with ethyl alcohol.
2. The animal is secured in a stereotaxic apparatus by holding the spinal process at T7.
3. The spinal process at T8 is removed using the rongeurs.
4. The rongeurs are carefully inserted into the intervertebral foramen (between T8 and T9), and bone from the base of the spinal process at T8 is removed.
5. After the spinal cord is exposed, a Hamilton syringe with a glass pipette is placed into a micropump's clamps and set up above the surgical area by mounting it onto a stereotaxic frame.
6. The cell suspension is absorbed from the tip of the pipette. (The amount of cells depends on your experimental design.)
7. Lower the syringe until the tip of the pipette touches the surface of the spinal cord.
8. Push the syringe further down using the micromanipulator, according to the depth decided upon (for example, 1mm deep from the surface).

9. Start the Nano injector to inject the cells. (Parameters, including the desired total amount of the cell suspension and the rate of cell injection, should be set before starting.)
10. After the injection is completed, the glass pipette is kept in place for a further five minutes to prevent the leakage of the cell suspension.
11. Pull up the syringe, and release the animal from the apparatus.
12. The muscle and the skin are sutured. The surgical area should be cleaned with ethyl alcohol.
13. Isoflurane is disconnected, and the animal is placed in a box with an infrared lamp (if the body temperature decreases) until it fully recovers from the general anesthesia.

## **6. Behavioral testing**

### **6.1. Plantar test**

The Plantar test is performed using a standard Ugo Basile test apparatus. The test consists of placing a rat in a transparent acrylic box, then a mobile infrared heat lamp is positioned underneath the targeted hind paw. A thermal radiant stimulus is then applied to the plantar surface, and the latency of the paw withdrawal response is measured automatically with the help of a photoelectric-sensitive device. The latency of the withdrawal response of each hind paw is determined before and after the spinal cord lesion is produced. The test is performed weekly after SCI during a given time period (from 2 to 6 months). Each paw is stimulated 5 times. Hyperalgesia in response to heat is defined as a decrease in withdrawal latency.

### **6.2. Open field locomotion (BBB)**

In order to properly assess behavioral outcomes following experimental SCI, a standardized locomotor rating scale was devised for testing rats in an open field. The Basso, Beattie and Bresnahan (BBB) locomotor rating scale was originally developed to assess mildly and moderately injured animal, then later tested on more severely injured rats. The sensitivity of the behavioral assessment can differentiate among various injury severities, such as hemisected, compressed or completely transected animal.

Two independent examiners study the locomotor ability of the test subject for approximately 4 consecutive minutes and rate the subject's locomotion using a 21-point scale (Basso et al., 1995). Following a surgical or chemical perturbation, the rats are subsequently tested beginning as early as 1 day post-treatment, with repeated testing routinely extending to 6-9 weeks post-treatment. The animals are tested once a week.

The BBB locomotor scale has a very wide rating range from no observable hindlimb movement (BBB score 0), to consistent plantar stepping and coordinated limb movement, consistent toe clearance, parallel paw position throughout the step cycle, and many other stages.

0. No observable hindlimb (HL) movement
1. Slight movement of one or two joints, usually the hip and/or knee

2. Extensive movement of one joint or extensive movement of one joint and slight movement of another joint
3. Extensive movement of two joints
4. Slight movement of all three joints of the HL
5. Slight movement of two joints and extensive movement of the third
6. Extensive movement of two joints and slight movement of the third
7. Extensive movement of all three joints of the HL
8. Sweeping with no weight support or plantar placement of the paw with no weight support
9. Plantar placement of the paw with weight support in stance only (i.e. when stationary) or occasional, frequent, or consistent weight-supported dorsal stepping and no plantar stepping
10. Occasional weight-supported plantar steps; no FL-HL coordination
11. Frequent to consistent weight-supported plantar steps and FL-HL coordination
12. Frequent to consistent weight-supported plantar steps and occasional FL-HL coordination
13. Frequent to consistent weight-supported plantar steps and frequent FL-HL coordination
14. Consistent weight-supported plantar steps, consistent FL-HL coordination, and the predominant paw position during locomotion is rotated (internally or externally) when it makes initial contact with the surface as well as just before it is lifted off at the end of the stance; or frequent plantar stepping, consistent FL-HL coordination, and occasional dorsal stepping
15. Consistent plantar stepping and consistent FL-HL coordination during gait, and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel to the body at initial contact and rotated at lift off
16. Consistent plantar stepping and consistent FL-HL coordination during gait, and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
17. Consistent plantar stepping and consistent FL-HL coordination during gait, and toe clearance occurs frequently during forward limb advancement; predominant paw position is parallel at initial contact and lift off
18. Consistent plantar stepping and consistent FL-HL coordination during gait, and toe clearance occurs consistently during forward limb advancement; predominant paw position is parallel at initial contact and rotated at lift off
19. Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, trunk instability; tail consistently up

20. Consistent plantar stepping and consistent coordinated gait, consistent toe clearance, predominant paw position is parallel at initial contact and lift off, trunk instability; tail consistently up
21. Consistent plantar stepping and coordinated gait, consistent toe clearance, predominant paw position is parallel throughout stance , consistent trunk stability; tail consistently up

## **7. Electrophysiology**

### **7.1. Protocol for brain slice preparation for patch-clamp recording**



Brain slice preparations are becoming increasingly popular among neurobiologists for the study of the mammalian central nervous system (CNS).

Brain slices are being used because they offer certain advantages over in vivo approaches to the study of the CNS. These are: 1) Rapid preparation, using relatively inexpensive animals (mouse, rat, guinea pig) where anesthetics are not necessary; 2) mechanical stability of the preparation, due to lack of a heart beat and respiration pulsations, which permits intracellular recordings for long periods; 3) simple control over the preparation's condition, where  $pO_2$ ,  $pCO_2$ , pH and temperature can be maintained; 4) direct visualization of the slice structure, allowing the accurate placement of the recording electrode in the desired sites; 5) slices have no blood brain barrier and thus their extracellular space is accessible to the perfusion medium and its content (ions, transmitters, drugs); 6) while simplified, the brain slice preparation maintains the structural integrity of the tissue, unlike cell cultures or tissue homogenates.

#### **7.1.1. Reagents**

##### Agar plate

Preparation: 4 g agar /100 ml Milli Q water brought to a boil at 100°C with a stirrer, then boil 10 min. Fill Petri dish with agar solution and solidify it at RT

Milli Q water (250 ml)

Isoflurane – inhalation anesthetic

1% Sodium-Pentobarbital (Sigma P3761 – intraperitoneal injection of anesthetic in physiological solution (0.9% NaCl) (100 mg/kg, i.p.)

NMDG-based isolation solution (500 ml) containing (in mM): 110 NMDG-Cl, 3 KCl, 23 NaHCO<sub>3</sub>, 1.25 Na<sub>2</sub>HPO<sub>4</sub>, 0.5 CaCl<sub>2</sub>, 7 MgCl<sub>2</sub>, 20 glucose, osmolality 290 mOsm/kg. Cold (4°C)!!!!!!

aCSF solution (250 ml) containing (in mM): 122 NaCl, 3 KCl, 28 NaHCO<sub>3</sub>, 1.25 Na<sub>2</sub>HPO<sub>4</sub>, 1.5 CaCl<sub>2</sub>, 1.3 MgCl<sub>2</sub>, 10 glucose, osmolality 305 mOsm/kg

Tissue adhesive 3M Vetbond No.1469SB

### **7.1.2. Equipment**

Gloves, latex

Four beakers (250 ml)

Isoflurane-specific vaporizers

Anesthesia syringe (5ml)

Perfusion syringe (20 ml)

Needle for pentobarbital (0.6x25 mm)

Needle for transcordial perfusion (1.20x40mm)

Surgical and dissection instruments:

Thumb forceps

Tissue forceps

Iris scissors

Microdissecting scissors

Steel scalpel and surgical blades

Hemostats

Spatula

Razor blade

Petri dishes with silicon

Solid agar cube 1cm<sup>3</sup>

Three pieces of filter paper

Vibration microtome (HM 650V, Thermo Scientific Microm, Walldorf, Germany)

Water bath at 34 °C

Gas tank (95% O<sub>2</sub>/5% CO<sub>2</sub>)

Brush

Strainer

Plastic tray

-20°C freezer

### **7.1.3. Procedure**

1. Turn on the water bath (34°C).

2. Make 600 ml isolation solution (prepare fresh daily, see above).

3. Prepare two isolation solution beakers (250 mL) for slice incubation.

4. Fill beaker for slice incubation with isolation solution. Place the beaker for slices in the water bath. Turn on O<sub>2</sub>/CO<sub>2</sub> (see above).
5. Fill vibratome chamber with Milli Q water.
6. Turn on the vibratome (HM 650V) to cool down the Milli Q water in the slice chamber to 3°C.
7. Freeze ~500 ml of isolation solution at -20°C for about 35 minutes.
8. Freeze Petri dishes with silicon for dissection of the brain.
9. Prepare a perfusion syringe (20 ml) with cold isolation solution.
10. Pour the Milli Q water from the vibratome chamber, then remove the isolation solution from the freezer and place ~250 ml in the vibratome chamber. Turn on O<sub>2</sub>/CO<sub>2</sub>.
11. Prepare 100 ml of cold isolation solution for transcatheter perfusion (40 ml) and brain isolation (60 ml).
12. Prepare one beaker with aCSF for maintaining the slices before recording.
13. Anesthetize the rat using isoflurane until asleep, then inject a sub-lethal dose of 1% sodium-pentobarbital intraperitoneally.
14. Put the animal in a plastic tray. Make an incision through the sternum and lift the rib cage to expose the heart. Release the heart from the surrounding tissue. The rib cage can then be either cut off or held back with hemostats. A needle is inserted through the left lateral ventricle at the 5:30 position. An incision can be made in the right atrium to allow the fluid to flow through. 40 ml is perfused to clear the blood from the animal and to replace Na<sup>+</sup>.
15. Rapidly decapitate the rat, dissect out the whole brain into Petri dishes with silicon with cold isolation solution.
16. Separate the cerebellum using a razor blade.
17. Glue the brain on the cutting disk using filtration paper and fix using 3M Vetbond tissue adhesive.
18. Transfer the brain to the vibratome chamber containing bubbling cold (3°C) isolation solution.
19. Cut 220µm slices on the vibratome at a frequency of 40 and an amplitude of 1. Then transfer each slice in a beaker of isolation solution for incubation.
20. Incubate for 30 minutes at 34°C in bubbling isolation solution. After incubation, the slices are kept at least 1 hour before recording in a holding chamber at room temperature in aCSF.

## **7.2. Patch-clamp recordings**

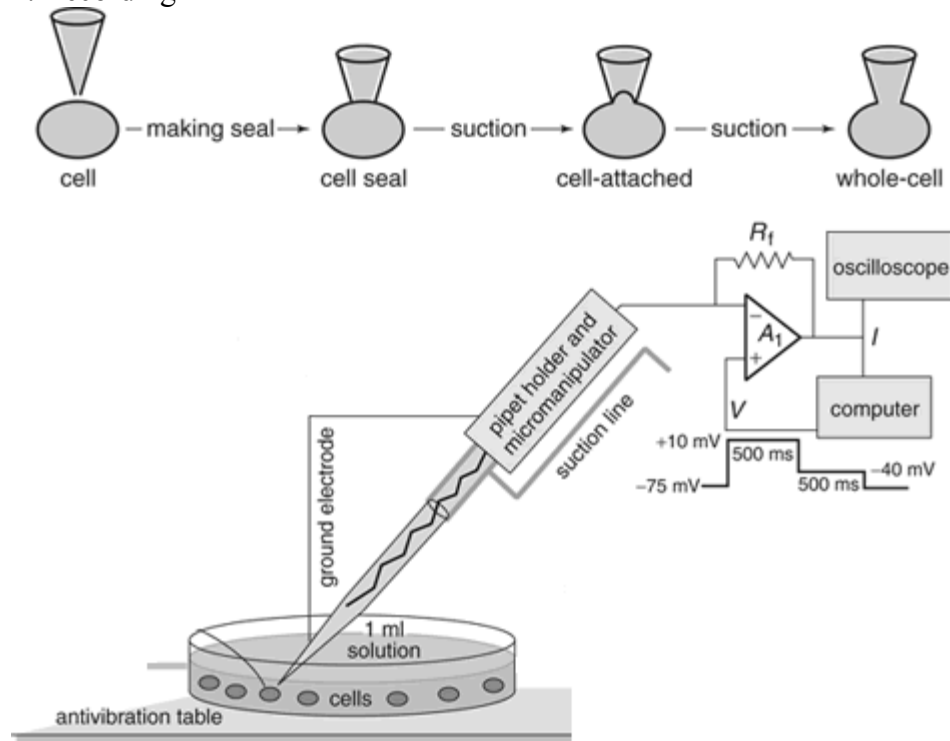
The patch-clamp technique is an electrophysiological method allowing the researcher to record the currents flowing through the cell membrane. The whole-cell configuration allows the recording of currents from the entire cell membrane.

### **7.2.1. Principles**

Recording of K<sup>+</sup> voltage-gated ion channels in the whole-cell configuration from neonatal neural stem cells differentiated *in vitro*

The recording itself consists of the following steps (see **Fig. 1**):

1. Producing and filling micropipettes
2. Forming a giga seal
3. Opening of the cell
4. Recording



**Fig. 1** Schematic image of the gigaseal formation and the recording process

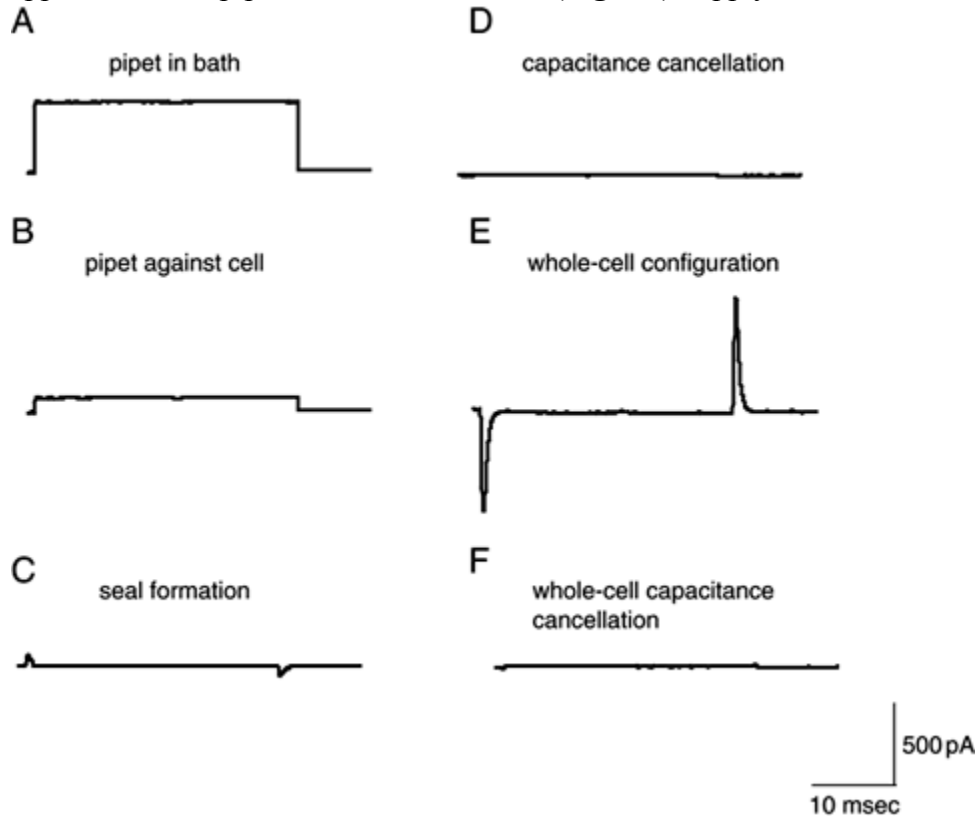
### 7.2.2. Producing and filling micropipettes

1. **A)** Switch on the horizontal micropipette puller and use programme 5 to produce microelectrodes with a tip resistance of  $\sim 6 \text{ M}\Omega$ .
- B)** Fill the microelectrode with the intracellular solution using a filling syringe.

### 7.2.3. Forming a giga-seal

- A)** Switch on the PatchMaster software, digital camera and Axiocam software, microscope light and the UV lamp. Switch on the perfusion pump, and when the chamber is filled with the bathing solution, place the cover-slip with cells into the chamber.
- B)** Find a suitable cell for recording on the cover slip, using the digital camera and the manipulators.
- C)** Place the filled electrode into the holder and connect it to the tubing that allows for pressure regulation.
- D)** Lower the electrode into the bath solution and check the electrode resistance in the amplifier window in the PatchMaster program (**Fig. 2A**).

**B)** Approach the cell with the electrode using the manipulators. Move the electrode to the cell surface and, at the same time, watch the baseline for changes in resistance, indicating the approach of the pipette to the cell surface (**Fig. 2B**). Apply suction to form a giga seal (**Fig. 2C**).



**Fig. 2** Forming a giga seal, opening of the cell and compensations.

Compensate C fast - compensate capacitance (C) and apply suction to open the cell (**Fig. 2E**). Finally, compensate C slowly (**Fig. 2F**) and switch to current-clamp mode to check the membrane potential.

#### 7.2.4. Recording

##### SOLUTIONS FOR PATCH-CLAMP

##### Bathing solution

STOCK SOLUTIONS	ml	final concentration (mM)
2M NaCl	122 ml	122 mM
1M KCl	6 ml	3 mM
0.5 M NaHCO <sub>3</sub>	112 ml	28 mM
0.1 M Na <sub>2</sub> HPO <sub>4</sub>	25 ml	1.25 mM
Glucose	3.6 g	10 mM

PhenolRed

Saturation by 95% O<sub>2</sub>/5% CO<sub>2</sub>

1M CaCl <sub>2</sub>	3.0 ml	1.5 mM
1M MgCl <sub>2</sub>	2.6 ml	1.3 mM

Intracellular solution (100 ml)

<u>STOCK SOLUTION</u>	<u>ml</u>	<u>final concentration (mM)</u>
1 M KCl	26 ml	130.0 mM
1 M CaCl <sub>2</sub>	0.1 ml	0.5 mM
1 M MgCl <sub>2</sub>	0.4 ml	2.0 mM
EGTA	0.20 g	5.0 mM
HEPES	0.24 g	10.0 mM

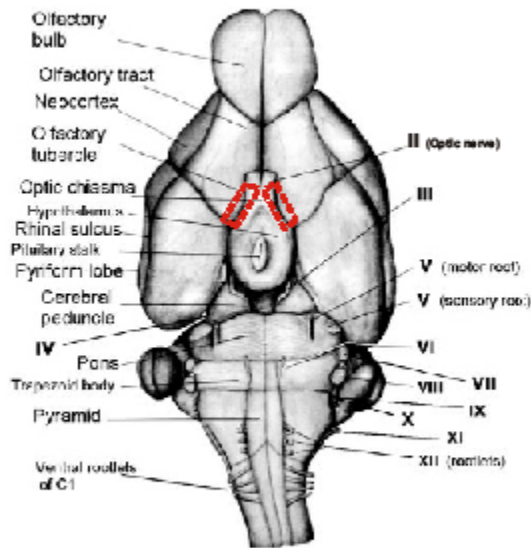
Adjust pH with KOH to 7.2.

**7.3. Preparation of CNS neurons for intracellular Ca<sup>2+</sup> measurements**

**7.3.1. Isolation of cells from the supraoptic nuclei**

- 2 adult (200-250g) Wistar rats for each experiment.
- Sacrifice the rats by a brief stunning on the lateral side of the rat in order not to damage the skull or induce bleeding, followed by decapitation with a guillotine
- This procedure should not last more than a couple of minutes. Remove the brain quickly from the skull and place it into Normal Locke's buffer (NL) containing: NaCl (140 mM), KCl (5 mM), Glucose (10 mM), MgCl<sub>2</sub> (1 mM), HEPES-Tris (10 mM), CaCl<sub>2</sub> (2 mM); pH 7.25; 295-300 mosmol/l<sup>-1</sup>)
- Using a dissection microscope, dissect two blocks of basal hypothalamic tissue (2 mm long, 1 mm thick).
- Transfer the dissected tissues immediately into 5 ml of Locke's buffer (37 C), supplemented with 1mg/ml DNase, 0.5 mg/ml Protease X and 0.5 mg/ml Protease-XIV for enzymatic dissociation for 45 min in the presence of O<sub>2</sub>.
- Prepare all of these enzymes in advance
- After the incubation period, wash out the enzymes with NL buffer.
- Continue with gentle mechanical trituration for 5-10 min.
- Plate the dissociated cells onto two glass bottom dishes.

## Dissection of the supraoptic nuclei



### 7.3.2. Loading of cultured cells with Fura-2,AM

Fura-2,AM (1 mM stock solution in anhydrous DMSO; 5  $\mu$ M final concentration)

0.02% Pluronic acid-F-127

Normal Locke's buffer (NL)

**Note: Keep dye solutions in the dark.**

- For a 5  $\mu$ M dye solution, add 20  $\mu$ l Fura-2 stock and 20  $\mu$ l 0.02% Pluronic F-127 to 4ml NL buffer.
- Replace the NL buffer with 2 ml of Fura-2 solution, allow the cells to load the Fura-2 solution for 45 min at room temperature (22-24  $^{\circ}$ C) in the dark.
- Wash out the excess Fura-2 dye with NL twice.
- Leave the dishes with the cells to stabilize in the dark for 30 min before  $Ca^{2+}$  measurements.

Fura-2-loaded cultures of SPC-01 and freshly isolated SON neurons plated onto glass bottom culture dishes for  $[Ca^{2+}]_i$  measurements will be placed in Locke's buffer and then mounted on a microscope stage. Fluorescence measurements of  $[Ca^{2+}]_i$  will be performed with a Zeiss Microscope Photometer System, based on an inverted microscope equipped for epifluorescence. With the fluorescence values corrected for the background and dark current,  $[Ca^{2+}]_i$  will be

calculated from the ratio between the 340 and 380 nm recordings.

### **7.3.3. Testing physiological and pharmacological agents that influence calcium signaling**

-to see the effect of high  $K^+$  (60 mM), glutamate (100  $\mu$ M), ATP (1  $\mu$ M), and caffeine (20 mM) in SPC-01 cell cultures.

-to see the effect of high  $K^+$  (60 mM), glutamate (100  $\mu$ M), vasopressin (100 nM), oxytocin (100 nM), and caffeine (20 mM) in freshly isolated vasopressin and oxytocin magnocellular neurons of the rat supraoptic nucleus.

## **8. Immunohistochemistry**

### **8.1. Identifying astrocytes**

#### **8.1.1. Chemicals and materials**

anesthesia:

sodium pentobarbital dissolved in saline solution (1%)

transcardial perfusion:

heparin diluted in saline (3000 IU/100ml)

4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 (PB)

fixation, cryoprotection:

4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4 (PB)

10%, 20%, 30% sucrose solutions in 0.1 M PB

immunohistochemistry:

0.01M phosphate-buffered saline, pH 7.4 (PBS)

blocking/diluent sol.: 5% ChemiBLOCKER (Millipore), 0.2% Triton in PBS

antibody: Cy3-conjugated mouse anti-GFAP (Sigma-Aldrich)

VECTASHIELD mounting medium (Vector Laboratories)

scissors, forceps, blade, scalpel

syringes, needles

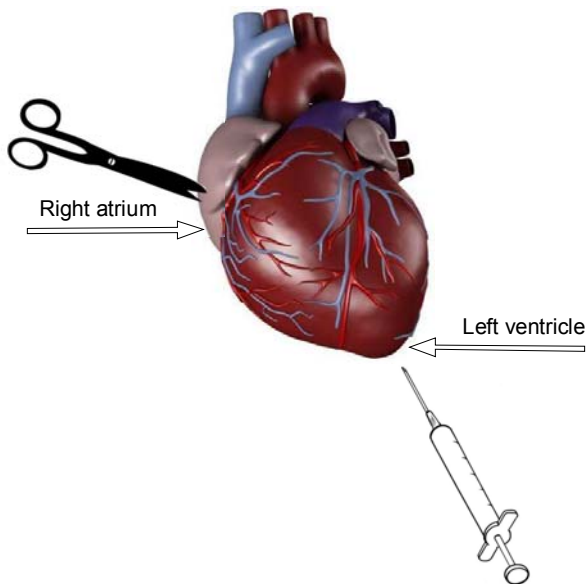
pipettes, culture dishes, microscope slides

#### **8.1.2. Procedure**

##### **8.1.2.1. Transcardial perfusion**

1. Anesthetize the animal via an intraperitoneal injection of **sodium pentobarbital**. (100 mg/kg, i.e. 0.3 ml for a 30g mouse).
2. Superficially cut the ventral skin with scissors, just below the xiphoid process.
3. Open the thoracic cavity by cutting the diaphragm from one lateral aspect to the other lateral aspect while avoiding cutting any visceral organs.

4. Carefully cut both lateral aspects of the rib cage, in a caudal to rostral direction, while avoiding the lung, heart and mammary arteries.
5. Fold back the ventral rib-cage surface and gently tape it into place to expose the thoracic organs.
6. Lacerate the **right atrial chamber** with scissors and carefully (but quickly) insert a needle attached to a heparinized-saline-syringe into the left **ventricular chamber**.
7. Begin **perfusion**:
  - a) heparinized saline (cca 20ml for a 30g mouse)
  - b) 4% PFA in PB (cca 20 ml for a 30g mouse)
8. Remove the infusion needle from the left ventricle and begin the dissection of the desired tissue (brain).



#### 8.1.2.2. Fixation, cryoprotection

1. Dissect the brain out of the skull and place in a bottle with paraformaldehyde solution (4% in 0.1M PB) for 3 hours.
2. Place the brain stepwise in solutions with a gradually increasing concentration of sucrose (10%, 20% and 30%) for cryoprotection. Proper saturation with sucrose is indicated by the brain sinking to the bottom of the container.
3. Use a cryostat (Leica CM1850, Leica Microsystems, Wetzlar, Germany) to prepare 30  $\mu\text{m}$  thick slices.

### 8.1.2.3. Immunostaining

Brain slices of transgenic GFAP/EGFP mice will be stained by the "**direct method**" with mouse anti-GFAP antibody conjugated to Cy3.

1. Wash slices in PBS.
2. Apply blocking solution (400  $\mu$ l) and incubate for 1 hour at RT.
3. Remove blocking solution and replace with antibody (400  $\mu$ l Cy3-conjugated anti-GFAP, diluted 1:200 in "diluent solution"). Incubate for 1 hour at RT.
4. Wash slices in PBS, 3 x 10 min.
5. Mount slices on a microscope slide using Vectashield mounting medium.