Immunostaining of fixed isolated Ventricular Cardiomyocytes

The Cardiomyocytes are obtained using the protocol "Standard isolation of ventricular cardiomyocytes ".

1. Equipment:

- Microscope
- 1000 µl, 100 µl, 10 µl and 2.5 µl pipettes
- Timer
- Stand for tubes
- Tweezers
- Dark chamber (for incubation with the secondary antibody)

2. Working materials:

- 12 well plate
- 50 ml tubes
- Towels
- 1000 µl and 100 - 200 µl pipette tips
- 10 µl stuffed pipette tips for antibodies
- Object carrier
- Cover glasses Ø 18 mm
- 1.5 ml reaction vessels
- Nail polish

3. Solutions:

Stock solution - 10x Perfusion buffer (1 L)

	Final concentration mM (1x buffer)	**MW (g/mol)**	**Amounts for 10x Buffer**
NaCl	120.4	58.4	70.3 g
KCl	14.7	74.6	11 g
KH_2PO_4	0.6	136.1	0.82 g
Na_2HPO_4 x $2H_2O$	0.6	177.99	1.1 g
$MgSO_4$ x $7H_2O$	1.2	246.5	3 g
HEPES	10	238.31	23.83 g
H_2O			1 L
pH-value			7.4 with NaOH

Working solution - 1x Perfusion buffer (500 ml)

	Final concentration mM (1x buffer)	**MW (g/mol)**	**Amounts for 1x Buffer**
10X Perfusion buffer			50 ml
$NaHCO_3$	4.6	84	0.195 g
Taurine	30	125.1	1.875 g
BDM	10	101.1	0.5 g
Glucose	5.5	180.2	0.5 g
H_2O			Up to 500 ml
pH-value			7.4

Coating cover glasses with laminin

Laminin stock solution (2 mg / ml)

18 mm or 24 mm cover glasses require 2 µl of stock solution

Spread the 2 µl on cover glass generously using a pipette tip.

4% paraformaldehyde (PFA) in 1x PBS (100 ml)

	Stock Concentration	**Final concentration**	**Amounts**
Paraformaldehyde (PFA)	100%	4%	4 g
10X PBS, pH 7.4	10X	1X	10 ml
H_2O			Up to 100 ml

PFA is toxic, storage at -20 ° C. Please refer to the detailed log "Production 4% PFA"!

• Defrost 4% PFA for 1 h at 4 °C. If after thawing white flakes are still in the Solution, then do not use the PFA (the pH value is not correct). Do not freeze the PFA then store it for longer than 1 day at 4 °C.

10 % Triton X-100 in 1x PBS

	Stock Concentration	Final concentration	Amounts
Triton X-100	100%	10%	1 ml
1x PBS pH 7.4			Up to 10 ml

Blocking buffer

	Stock Concentration	Final concentration	Amounts
10 % Triton X-100 in 1x PBS	10%	0.2%	1 ml
BCS (Bovine calf serum)	100%	10 ml	5 ml
1x PBS pH 7.4			Up to 50 ml

Always prepare buffer fresh, store up to 2 days in fridge.

1x PBS, pH 7.4 (for washing the cells) - purchased solution

Mounting Medium with or without DAPI - purchased solution

- **Prolong Gold Antifade reagent (Invitrogen)**

  Cat #P36934 (without DAPI)

  Cat #P36935 (with DAPI)

4. Protocol:

!! Caution: The cells, whether live or already fixed, must never be allowed to dry in this protocol.

4.1. Prepare the 12 well plate (at room temperature)

• Add the appropriate number of Ø 18 mm cover glasses into the 12 well plate

• Before the cells are placed on the cover glass, the cover glass must be coated with laminin

• Apply 2 µl of laminin

• Distribute the laminin with a pipette tip on the cover glass

• Incubate for 15 - 30 min (This is an indication from the manufacturer, an incubation time is advantageous for the immunostaining)

4.2. Plate cells on cover glass (at room temperature)

After the optical evaluation of the cells and setting the right optical Cell density (see protocol: "Standard isolation of ventricular cardiomyocytes")

• Add 200 µl of cell suspension to each 18 mm laminated glass. !! Attention: only use 1 ml pipette tips

• !! Caution: Do not run the cell suspension over the top of the lid

• Let the cells settle for 10-30 min

• !! Caution: Do not displace the plate so that the cells settle at rest and adhere to the cover glass surface using the laminin.

4.3. Fixation of cells and blocking (at room temperature)

• Add 500 µl Perfusion Buffer per well

• Aspirate 700 µl (200 µl Cell suspension + 500 µl Perfusion buffer)

• Carefully apply (do not disturb cells) with 1 ml pipette, 3 drops of 4% PFA / well (Wait 5 min)

• Add 500 µl blocking buffer per cover glass / well with a pipette to the cells

• Remove the remaining solution completely (keeping the plate slightly at an angle)

• Add 500 µl of fresh blocking buffer per cover glass / well to the cells

The cells are now blocked, i.e All nonspecific binding sites are occupied by the BCS. This blocking prevents nonspecific binding of the antibodies and the background signal is reduced.

• Incubate in blocking buffer for at least 1 h (RT) to overnight (If overnight, then store the plate at 4 ° C)

4.4. Incubation with primary antibody (at 4 ° C)

• Dilution of primary antibodies:

⇨ Check table "primary Antibody list "
⇨ The antibody is diluted in blocking buffer

• 500 µl per cover glass / well

• Completely remove the buffer (blocking buffer) from the well (hold the plate at an angle)

• Carefully apply the diluted antibody to the cells

• Incubate with the primary antibody overnight at 4 ° C

4.5. Washing with Blocking Buffer (at room temperature)

• Remove the supernatant completely from the well (plate at angle)

• Wash (2x) twice for about 1 min with 500 µl blocking buffer and immediately remove it

• Wash 1x for 10 min with 500 µl blocking buffer

4.6. Incubation with secondary antibody (at room temperature, in the dark)

• Dilution of primary antibodies:

⇨ Check table "secondary Antibody list "
⇨ The antibody is diluted in blocking buffer

• 500 µl per cover glass / well

• Completely remove the buffer (Primary antibody) from the well (hold the plate at an angle)

• Carefully apply the diluted antibody to the cells

• Incubation with the secondary antibody for 2 - 3 h (in the dark)

4.7. Washing with PBS (at room temperature)

• Remove the supernatant completely from the well

• Wash twice for about 1 min with 500 µl of 1x PBS, immediately remove it (hold the plate at an angle)

• Wash 1x for 10 min with 500 µl of 1x PBS, the wash cycle can be extended to 30 minutes

• This supernatant does not have to be removed

4.8. Mount cells (at room temperature)

Fetch Mounting medium in time from the freezer, approx. 15 min before

• Label the slide (with date, primary and secondary Antibodies, specimen slides, possibly different dilutions)

• Place 1 drop of mounting medium on the slides for each cover glass

• Using tweezers, gently remove the cover glass from the 1x PBS

• Carefully drain the remaining liquid on a cloth

• Place cover glass containing the cells on the slide with the mounting medium, cells must be facing mounting medium

(Try to avoid air bubbles by placing at an angle and slowly lowering cover glass unto slide)

• Place the slides back into the darkroom and let them dry overnight

!! Attention: do not dry at 4 ° C, the mounting medium will not harden

• On the next day, seal the cover glass edge with nail varnish

• Stored samples at 4 ° C

5. Companies + Ordering numbers:

Consumables	SAP / Company	Ordering number
50 ml tubes	SAP / Sarstedt	4003394
Super frost plus slides	SAP / Menzel	4008533
1000 µl Pipette tips	Brandt	712320
100 – 200 µl Pipette tips	Brandt	712315
10 µl Filtered Pipette tips	Star lab	S1121-3810
Cover slips Ø 18 mm	Roth	LH 23.1
12 Well plate	SAP / Greiner bio one	4022860
1.5 ml Eppendorf Reaction tubes	SAP / Eppendorf	4004646
Chemicals	**Company**	**Ordering number**
Sodium Chloride, 1 Kg	Roth	HN00.2
Potassium Chloride, 500 g	Roth	6781.3
di-Sodium Hydrogen Phosphate x $2H_2O$, 500 g	Roth	4984.2
Potassium Hydrogen Phosphate, 250 g	Roth	3904.2
Magnesium Sulfate x $2H_2O$, 500 g	Roth	P027.1
HEPES, 500 g	Roth	9105.4
Sodium Hydroxide solution (2 M), 1 L	Roth	T135.1
Sodium bicarbonate, 500 g	Roth	HN01.1
Taurine (2-aminoethanesulfonic acid), 500 g	Roth	4721.2
BDM (2 3-butanedione monoxime), 25 g	Sigma	B0753
Glucose, 250 g	Roth	HN06.1
Triton X - 100, 100 ml	Sigma	T9284
1x PBS pH7,4; 500 ml	Invitrogen	10010-015
BCS (Bovine Calf Serum), 100 ml	Fisher Scientific	SH30073.02
Paraformaldehyde (PFA)	Sigma	158127
Laminin, mouse, 1 mg	BD Biosciences	354232
10 x PBS, pH7,4; 500 ml	Invitrogen	AM9624
1 x PBS, pH 7,4; 500 ml	Invitrogen	10010-015
Mounting medium prolong gold anti-fade reagent with DAPI	Invitrogen	P36935
Mounting medium prolong gold anti-fade reagent WITHOUT DAPI	Invitrogen	P36934