

# Di-8-ANEPPS loading of neurons - voltage

## 1- Stock solution of Di-8-ANEPPS :

**Dye:** Di-8-ANEPPS – 5mg [Invitrogen D-3167]

**Storage:** -20°C – Protect from light

**MW:** 592.8784

**Open** the 5mg vial.

Add 1.185ml of DMSO.

Vortex

Aliquot by 10µl in ependorfs (0.5ml).

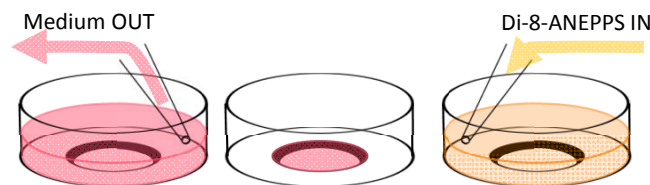
**Storage:** up to 1 year in the freezer at -20°C – Protect from light

## 2- Loading of the cells:

**On the day of experiment:**

**1** - Open a 10µl Di-8-ANEPPS aliquots (the number of aliquots varies with the final concentration you want to achieve). Add it to 2.5ml of extracellular solution and vortex.

**2** - Remove most of the medium in the culture dish: pipette the medium out from the corner between the outside wall and the bottom of the dish



**3** - Add 1ml of Di-8-ANEPPS containing solution

**4** - Allow the cell to rest for 20 min in the dark at room temperature (membrane loading), wash the content with extracellular solution and use the cells immediately for recordings. If the cell is at 37°C give 10 min loading time. Keep in mind that at 37 °C the dye will translocate to intracellular compartment with time.

## 3- Reading:

For visualisation of intracellular calcium changes, the Di-8-ANEPPS is excited at  $\lambda=470\text{nm}$  (blue) and the emission reading is at  $\lambda=630\text{nm}$  (red)

**Note:** Careful, Di-8-ANEPPS has a short life span and degrades quickly with light. Use short exposition time with high sensitivity of the camera. The dye would last for a total 20s at this concentration (this gives 400 frames of 50ms exposure).

# Fura-2 AM loading of neurons - calcium

## 1- Stock solution of Fluo4-AM:

**Dye:** Fura-2 AM – Special packaging (20 x 50µg) [Invitrogen F-1221]

**Storage:** -20°C – Protect from light

**MW:** 1001.86

**Open** two aliquots of 50 µg.

Add 50µl of DMSO in each (Concentration 1mM).

Vortex.

Aliquot by 10µl in 10 ependorfs of 1.5ml volume.

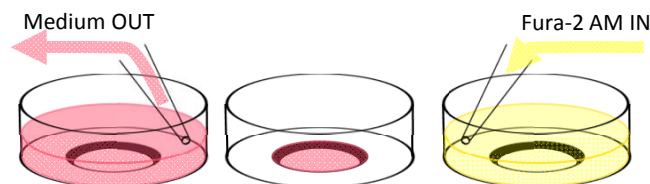
**Storage:** up to 3 months in the freezer at -20°C – Protect from light

## 2- Loading of the cells:

**Per culture dish:**

1 - Open one 10µl Fura-2 AM aliquot, add 1ml of extracellular solution and vortex (Final concentration in 1ml medium is 10µM).

2 - Remove most of the medium in the culture dish: pipette the medium out from the corner between the outside wall and the bottom of the dish



3 - Add 1ml of Fura-2 AM containing solution

4 - Allow the cell to rest for 40 min in the dark at room temperature (loading).

5 - Rinse well with medium to remove the Fura-2 AM containing solution and replace it by external solution. Allow the cells to rest in the dark for 40 min (de-esterification)

The de-esterification interval can be used to load the membranes with Di-8-ANEPPS.

## 3- Reading:

For visualisation of intracellular calcium changes, the Fluo4 is excited at  $\lambda=480\text{nm}$  (light blue) and the emission reading is at  $\lambda=520\text{nm}$  (green)

# Fluo4-AM loading of neurons - calcium

## 1- Stock solution of Fluo4-AM:

**Dye:** Fluo4-AM – Special packaging (10 x 50µg) [Invitrogen F-14201]

**Storage:** -20°C – Protect from light

**MW:** 1096.95

**Kd for Ca<sup>2+</sup> in buffer:** ~335 nM

**Open** two aliquots of 50µg.

Add 76µl of DMSO in each (concentration is 0.9mM).

Vortex

Aliquot by 10µl in 15 ependorfs of 1.5ml volume.

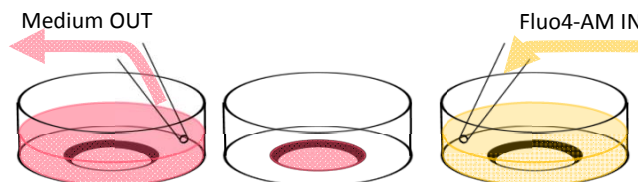
**Storage:** up to 3 months in the freezer at -20°C – Protect from light

## 2- Loading of the cells:

**Per culture dish:**

1 - Open one 10µl Fluo4-AM aliquot, add 1ml of extracellular solution and vortex (Final concentration in 1ml medium is 9µM).

2 - Remove most of the medium in the culture dish: pipette the medium out from the corner between the outside wall and the bottom of the dish



3 - Add 1ml of fluo4-AM containing solution

4 - Allow the cell to rest for 40 min in the dark at room temperature (loading).

5 - Rinse well with medium to remove the Fluo4-AM containing solution and replace it by external solution. Allow the cells to rest in the dark for 40 min (de-esterification)

The de-esterification interval can be used to load the membranes with Di-8-ANEPPS.

## 3- Reading:

For visualisation of intracellular calcium changes, the Fluo4 is excited at  $\lambda=480\text{nm}$  (light blue) and the emission reading is at  $\lambda=520\text{nm}$  (green)

# Retrograde labelling of airways neurons

## 1- Stock solution of labelling dye:

**Dye:** "Dil" – DiI<sub>C<sub>18</sub></sub>(3) (1,1'-dioctadecyl-3,3,3',3'-tetramethyl-indocarbocyanine perchlorate) [Invitrogen D282]

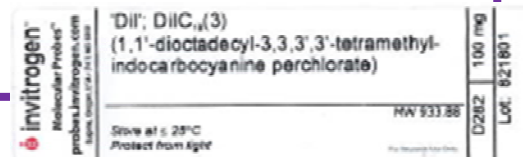
**Storage:** ≤ 25°C – Protect from light

**MW:** 933.88

**A** – Dilute the Dil powder in pure sterile ethanol to obtain a concentration of 25mg/ml (4ml Ethanol for 100mg Dil).

**Storage:** up to one year on the shelf - ≤ 25°C – Protect from light

**B** – On the day of dosing mix Dil aliquots with 0.9% sterile Saline: 10µl in 0.4ml sterile saline per animal to stain. Mix thoroughly.



## 2- Procedure:

**Animal:** Protocol for Young adult male guinea pigs (300-350g)

**Anaesthesia:** none

**Preparation:** - Restrain the Guinea pig head up. Inclined 30° backward  
- Intranasal application using a Gilson pipette and a fine tip.

**Administration:** - Mix again the Dil aliquot in 0.9% sterile saline immediately prior instilling the dye (vigorous shake).

- Instil 0.1ml/100g animal of the Dil-Ethanol/Saline emulsion into the lungs intra-nasally. For a 300<sup>+</sup>g guinea pig, use 0.4ml.

**Caution:** the Dil stock solution stains very easily skin or any other surface. Staining can be removed with 70% ethanol.

## 3- Reading:

Dil travel along the neuron membrane at a speed of 6mm a day. The labelling last up to 60 days. The dye can stay in situ up to one year.

Sacrifice the animals 14 days later to isolate the cells

For visualisation of labelled neurons, the Dil is excited at  $\lambda=540\text{nm}$  (green) and the emission reading is at  $\lambda=600\text{nm}$  (orange)