Unroofing mammalian cells for AFM

Veronika Cencen[1]

[1]EPFL - EPF Lausanne

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Working

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Veronika Cencen

ABSTRACT

Unroofing mammalian adhesive cells for the purpose of AFM imaging

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://www.parksystems.com/images/products/nx-bio/Use_of_the_unroofing%20technique_for%20atomic_force_microscopic_imaging_of_the_intra-cellular_cytoskeleton_under_aqueou.pdf

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

Some chemicals in this protocol are harmful and require additional PPE

MATERIALS

NAME	CATALOG #	VENDOR
Potassium hydroxide	View	P212121
Glutaraldehyde EM Grade 25%	G5882-50ML	Sigma Aldrich
Magnesium Chloride	AC223210010	Fisher Scientific
Sodium phosphate monobasic monohydrate	S9638	Sigma Aldrich
EGTA	View	Sigma Aldrich
DTT	D0632	Sigma Aldrich
HEPES	H6147	Sigma Aldrich
KCl		
NaCl	53014	Sigma Aldrich
HCl	20248.295	
Water, deionized	WC8800.SIZE.4L	Bio Basic Inc.
AEBSF	A-540	Gold Biotechnology
Poly-L-Lysine Solution, 10X, For 100-200 Slides	AR0003	Boster Bio
Calcium chloride	1.02378.0500	Merck Millipore
Paraformaldehyde	15710	Electron Microscopy Sciences
Sodium hydroxide	1064981000	Merck Millipore
EDTA	AM9261	Invitrogen - Thermo Fisher

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Molecular Biology Grade Water	10154604	Fisher Scientific
NaCl	53014	Sigma Aldrich
KCl	1049360250	Merck Millipore
Magnesium Chloride	AC223210010	Fisher Scientific
Sodium phosphate monobasic monohydrate	S9638	Sigma Aldrich
Glucose	G8270	Sigma Aldrich
HEPES	H6147	Sigma Aldrich
Calcium chloride	1.02378.0500	Merck Millipore
EDTA	AM9261	Invitrogen - Thermo Fisher
EGTA	View	Sigma Aldrich
AEBSF	A-540	Gold Biotechnology
DTT	D0632	Sigma Aldrich
Paraformaldehyde	15710	Electron Microscopy Sciences
Glutaraldehyde, 25% solution	GC3870.SIZE.500ml	Bio Basic Inc.

SAFETY WARNINGS

BEFORE STARTING

Read the purpose of unfamiliar chemicals

Buffers

1 00:30:00

Mix the following at 1 g powder in 10 ml

Molecular Biology Grade Water
by Fisher Scientific
Catalog #: 10154604

NaCl
by Sigma Aldrich
Catalog #: 53014

KCl
by Merck Millipore
Catalog #: 1049360250

Magnesium Chloride
by Fisher Scientific
Catalog #: AC223210010

Sodium phosphate monobasic monohydrate
by Sigma Aldrich
Catalog #: S9638

Glucose
by Sigma Aldrich
Catalog #: G8270

HEPES
by Sigma Aldrich
Catalog #: H6147

Calcium chloride
by Merck Millipore
Catalog #: 1.02378.0500

2 00:30:00 Prepare 1g/10mL at pH 8 of

EDTA
by Invitrogen - Thermo Fisher
Catalog #: AM9261

EGTA
by Sigma Aldrich
View

3 00:15:00 On the day of intended use, also prepare 1g/10ml

AEBSF
by Gold Biotechnology
Catalog #: A-540

DTT
by Sigma Aldrich
Catalog #: D0632

step case

If AEBSF and DTT ar not used that day

Store at -20 °C

SAFETY INFORMATION
read the safety data of both, prepare with appropriate caution

4 00:15:00 Dilute depending on method of storage

Paraformaldehyde
by Electron Microscopy Sciences
Catalog #: 15710

Glutaraldehyde, 25% solution
by Bio Basic Inc.
Catalog #: GC3870.SIZE.500ml

Glutaraldehyde is better for crosslinking and structure preservation, paraformaldehyde is faster and better for thick tissue

5 01:00:00

Create buffers "HEPES-free Ringer;s", "Ringer's" and "Ca++ free Ringer's" by

1. adding "check V final" from the attached table into a beaker
2. Adjusting the pH with KOH TO 7.4 while mixing on a magnetic plate
3. Adding DI water to the final volume of 100mL

unroofing stocks (1).xlsx

If pH gets too high, adjust with HCl, but do not surpass the final volume

SAFETY INFORMATION

Make sure to add calcium at the end
Wear protective equipment when using strong bases

step case

If buffers are not needed that day
These buffers can be stored in the fridge

6 25 °C 00:15:00

Prepare KHMgE buffer. If not using that day, leave out the DTT and add before use go to step #1

SAFETY INFORMATION

Use caution with AEBSF; check if other protease inhibitor is available. Avoid preparing stock as it is unstable in water

EXPECTED RESULT

AEBSF should preserve protein structure better due to protease inhibition
DTT is a redox reagent to stabilize protein

Cell prep

7 48:00:00

Prepare cells on cover slips

PROTOCOL

Basic Cell Culture Maintenance: Splitting Cells
by David Ellison,
Oregon Health & Science University

PREVIEW RUN

7.1 Confirm that cells are at least 80% confluent by microscopy.

7.2 Warm complete DMEM in 37°C water bath and thaw trypsin at room temperature.

7.3 Sterilize the biosafety cabinet with 10% bleach for 20 minutes. Spray down the biosafety cabinet with 70% ethanol and use UV light for 15

minutes as a secondary decontaminant.

7.4 Aspirate the media from the flask using a sterile autoclaved glass pipette. **Do not touch the cells with the pipette.**

To avoid touching cells, is best to tilt the flask and gently remove media from a corner.

7.5 Careful not to disturb cells adhered to the wall with the flow, add 5 mL PBS.

7.6 Gently swish PBS over cells to wash off the media by gently rocking it over the cells on the flask wall.

7.7 Quickly, aspirate PBS out. Cells will detach if PBS is left on them for too long.

7.8 Add 3 mL trypsin and gently rock the flask to cover cells.

Trypsin is harmful to the cells. Pay special attention that cells are not in trypsin longer than one minute.

7.9 Incubate flask at 37° for 30 seconds to 1 minute until cells start lifting off.

00:00:30 Incubate cells with trypsin.

During incubation, quickly prepare and label a 50 mL falcon tube for the next steps.

7.10 ***Remove cells from incubator and quickly, smack!*** until cells are no longer adhered to the wall.

7.11 Add 7mL warm complete medium, rinsing multiple times by pipetting up and down, to neutralize the trypsin reaction. Pipette towards cell side of flask walls.

Trypsin may destroy your cells if you are not fast enough to neutralize it.

7.12 Transfer ALL contents/cells to a 50 mL falcon tube.

7.13 Spin down 3,000 rpm for 5 minutes.

00:05:00 Spin down cells at 3,000 rpm.

7.14 While spinning, clean surfaces with EtOH and label new flasks, noting the +1 passage number and dilution.

7.15 Aspirate media from falcon tubes with cells; make sure to not disturb the pellet.

7.16 Add 10 mL media to pellet and pipette *violently* up and down.

7.17 Add 8 mL fresh media to new flask, then 2 mL of resuspended cells. (2:10 dilution)

Or 9 mL fresh media, then 1 mL resuspended cells. (1:10 dilution)

7.18 Gently shuffle, ensure even dispersal, and return the fresh flask to incubator.

7.19 Count and plate cells with leftovers after splitting, then move on to transfection or other experiments tommorow.

Unroofing

8 00:05:00

Wash fixed cells once with (HEPES)-based Ringer's solution and then with **Ca++-free Ringer's solution**.

EXPECTED RESULT

HEPES should improve appearance of cell health

9 00:05:00

Soak the cover slips for about 10 s in poly-lysine solution **(0.5 mg ml−1poly-lysine dissolved in Ca++-free Ringer's solution)**, wash three times for a few seconds each in hypotonic Ringer's solution prepared by mixing **one part of Ringer's solution with two parts of distilled water (DW)**.

EXPECTED RESULT

This induced cell swelling, which enabled the cells to burst easily following ultrasonic stimulation.

SAFETY INFORMATION

Keep sonicator power below "micropore" max

10 00:05:00

Immediately after immersing in hypotonic solution, expose cells to a small bubble jet by weak ultrasonic vibration in isotonic KHMgE buffer

go to step #6

Use a micro-probe

Model 3000

Ultrasonic Homogenizer

BioLogics 0-127-0001

The Model 3000 delivers up to 300 watts of ultrasonic disruption and includes an integrated Sound Abating Chamber to reduce cavitational sound emitted during processing. The clear Plexiglas door permits viewing of the sample while protecting the operator against accidental splashing. An access port for tubing is also provided for use with Cup Tips and the Continuous Flow Chamber. The Timer and Pulser function increase precision in sample processing.

11 **00:05:00**

Wash briefly in fresh KHMgE buffer

12 Fix for 10 min with 0.5% glutaraldehyde and 1% paraformaldehyde in KHMgE buffer. **00:10:00**

13 **00:05:00**

Wash fixed cells twice with KHMgE buffer and use for imaging and AFM