

Appendix B

Cell, Cleaning and Surface Chemistry Protocols

Protocols presented:

- N2a cell plating
- N2a cell transfection
- N2a cell media
- N2a cell media-clear
- Intracellular Fluid
- Extracellular Liquid
- Coverslip cleaning
- Cell Lysis
- Membrane patch preparation
- N2a cell top membrane adhesion
- Linking carboxylated beads to APTES coated coverslip
- APTES coating glass coverslips

N2a cell plating protocol

L. Wade

April 13, 2009

This protocol is for splitting flasks of N2a cells and for plating cells into new culture dishes.

Basics:

Sterilize everything with 70% ethanol. Then put into hood

Spray hands too....every time you touch something outside the sterile box.

Wear short sleeves or roll up long sleeves.

Stay sterile

Toss anything that touches anything else

Fluids used:

TrypLE express (Gibco 12605)

This is basically trypsin. It is used to separate the cells from the flask in which they've been growing.

N2a Cell Media Protocol for 500 ml solution:

- | | |
|---|----------|
| a. DMEM, high Glucose, 4mMl-glutamine (Gibco 11965-092) | 222.5 ml |
| b. Fetal Bovine Serum, Qualified (Gibco 26140-079) | 50 ml |
| c. Optimem1 | 222.5 ml |
| d. Pen/Strep (100x) (Mediatech 30-009-CI/Gibco 15140-122) | 5 ml |
- note this is a mix of the antibiotics Penicillin and Streptomycin

Add together, stir, and then 0.22 μ m filter into sterile 500 ml container.

I checked pH: 7.42 without titration.

Flask Splitting protocol:

Prepare three 50 ml centrifuge tubes.

Add 5 ml of TrypLE (avoids contamination of main bottle) to one of the tubes.

Transfer 50 ml of N2a growth media into the second tube.

Spray and pass the third tube into the sterile hood to hold cells later in protocol.

Separate cells from flask.

Evacuate all serum from flask.

Use 5 ml pipette to add a couple ml of N2a cell media.

Roll this around flask and then evacuate this media.

Add 2-3 ml of trypsin, roll it around flask and then set flask in incubator for 2 to 4 minutes.

Add 10 ml of N2a growth media to flask.

Suck back into 10 ml pipette, then back out then back in.

Repeat this a total of 3 to 4 times.

Suck up the ~12 to 13 ml of cells-in-media solution and put into the previously sterilized empty 50 ml bottle.

Suck about 1 ml of this media (for 10:1 split...or ~2.5 ml for 5:1 split) and put into a new sterile flask.

Add 13 ml of N2a cell media to this new flask.
 Label flask with the passage number, the date of plating, your name, the cell type.
 Place new flask in incubator.
 Discard old flask and TrypLE containing 50 ml bottle.

Notes: p30 is the end of a cell line's useful life. More than p25 is pretty old, and p28 and more is aged severely. My experience is that old cell lines are less vital and express (the desired) proteins at much lower levels than new cell lines do.

Plate cells into new culture dishes:

Pass new culture dishes into the sterile hood after spraying liberally with ethanol.
 Calibrate the cell density in the 50 ml tube left over from splitting the cells from old flask:
 Turn 50 ml tube up and down a couple times to mix the cells in the media.
 Pipette out ~100 μ l of this solution.
 Wick into hemacytometer with coverslip.
 I use one made by Hausser Scientific, model 3500, 'Levy Hemacytometer, improved Neubauer.' Each group of 16 squares has a volume of 0.1 μ l.
 Count the number of cells in each of the four 16 grid locations.
 Count cells on the top and lefthand lines but not those on the bottom or righthand lines.
 Divide the total by four.
 The density in the 50 ml flask is therefore this number times 10,000 per ml.
 For example: a total count of 236 mean that there were an average of 59 cells per 16 grid location and that the flask has a density of 590,000 cells per ml of media/cell solution.

We want to plate about 90,000 cells into each new culture dish. So in the case described above we want to put $90,000 \text{ cells} / 590,000 \text{ cells/ml} = 0.15 \text{ ml}$ of solution pipetted into each culture dish (this is for large dishes (50-60 mm dia)). For my teflon 35 mm dishes I plated 25,000 cells.

For my 35 mm teflon dishes with 20 mm coverslips try 10,000 cells.

Also add 4 ml of N2a cell growth media into each culture dish.

Gently swish cells in a figure 8 until well mixed.

No opaque clump in center should be visible.

Put into incubator.

Dispose of everything and spray everything in sterile hood with ethanol including the suction tube.

Longevity

Cells in culture dish ready for transfection in ~1 day.

Cells in flask will live for 3-4 days and then need to be split again.

Cell transfection protocol

L. Wade

April 13, 2009

This protocol is for transfecting DNA plasmids into N2a cells.

Basics:

1. Sterilize everything with 70% ethanol. Then put into hood.
 - a. Spray hands too....every time you touch something outside the sterile box.
 - b. Wear short sleeves or roll up long sleeves.
2. Stay sterile.
 - a. Toss anything that touches anything else.

Fluids used:

DMEM (Gibco 11965)

ExpressFect Transfection Reagent (Denville Scientific)

DNA Plasmids to be transfected

N2a cell growth Media (only needed for final step)

Transfection solution preparation:

- 1) Pass one ~1ml centrifuge tube into the sterile hood for each dish being prepared, plus One extra.
- 2) Transfer 100 μ l DMEM into one centrifuge tube for each dish to be prepared.
- 3) Add plasmid to each centrifuge tube for each dish in the appropriate amount:
 - a. 75 ng of mCherry labeled lyn kinase (membrane localized protein) is 0.46 μ l (160 ng/ μ l).
 - b. 500 ng of α 4 with GFP or β 2 with or w/o GFP. Density is 100 ng/ μ l so for these transfer 5 μ l into each tube.
- 4) Prepare 'master mix':
 - a. Into extra tube transfer 100 μ l of DMEM plus 8 μ l of Expressfect for each 50 mm dish being prepared (4 μ l for a 35 mm dish).
 - b. Gently tap bottom of this master mix several times to mix.
- 5) Pipette 104 μ l of 'master mix' into each centrifuge tube (one per dish).
- 6) Vortex and then let sit for 15-20 minutes.

Transfect culture dishes:

- 1) Pipette ~3ml media from culture dishes to be transfected.
 - a. Leaves about 1.5 mm deep media in each dish.
 - b. Put removed media into a 50 ml tube for future disposal.
- 2) Pipette all liquid from one centrifuge tube (about 205 to 210 μ l each) and transfer into a culture dish.
- 3) Gently swirl each of the dishes to mix fluids.
- 4) Place all transfection culture dishes into incubator and wait 4 hours.
- 5) Wipe everything down with ethanol mixture (70%)
- 6) After four hours evacuate transfection mixture out of each dish and immediately add ~4 ml of N2a cell media.
- 7) Swirl some and suction up again.
- 8) Once again immediately add 4 ml of N2a cell media and then place sealed dish in incubator.

- 9) Dish will be ready to image in 48 hours (optimally).
- Can be imaged as early as 24 hours with low levels of protein expression.
 - 36 to 60 hours is the optimal range.

N2a Cell Media Protocol

500 ml solution	
DMEM, high Glucose, 4mMl-glutamine (Gibco 11965-092)	222.5 ml
Fetal Bovine Serum, Qualified (Gibco 26140-079)	50 ml
Optimem1	222.5 ml
Pen/Strep (100x) Mediatech 30-009-CI/Gibco 15140-122)	5 ml
(note this is a mix of the antibiotics Penicillin and Streptomycin)	

Add together, stir, and then 0.22 μ m filter into sterile 500 ml container.

I checked pH today: 7.42 without titration.

N2a Cell Media-clear

445 ml of DMEM without phenol red
 5 ml of penicillin streptomycin (we have aliquots)
 50 ml of FBS

Intracellular fluid

Rev 2 with recommended changes by Fraser Moss on 9 January 2009:

<u>mM</u>	<u>Compound</u>	<u>Fw</u>	<u>100 ml (1X)</u>	<u>150ml (1X)</u>
130	KCl	74.55	969.2 mg	1.454 g
6	MgCl ₂ .6H ₂ O	203.3	122.0 mg	183 mg
5	EGTA	380.4	190.2 mg	275.3 mg
10	HEPES	238.3	238.3 mg	357.5 mg

Titrate the pH of this solution to ~7.4 using **KOH** NOT NaOH.

Also check the osmolarity of this solution. Typically mammalian cells in culture will be in media of 296mOsm/kg. Try and get your solutions in this ballpark (+/- 10% max). Add glucose to increase and water to decrease. Check that the concentrations above have not been changed if water is added.

The solution below includes ATP, which is not necessary for today's work (membrane patches) as my cell is not functioning:

<u>mM</u>	<u>Compound</u>	<u>Fw</u>	<u>100 ml (1X)</u>
110	K-gluconate	234.25	2.577 g
20	KCl	74.55	149.1 mg

1	MgCl ₂ .6H ₂ O	203.3	20.3 mg
5	EGTA	380.4	190.2 mg
5	MgATP	507.2	253.6 mg
10	HEPES	238.3	238.3 mg

Titrate the pH of this solution to ~7.4 using **KOH**.

For whole cell patch clamping use:

From Nashmi-JNeuro-2007

Whole-cell recordings were performed with glass electrodes (2–5 MΩ) filled with an internal solution [88 mM KH₂PO₄, 4.5 mM MgCl₂, 0.9 mM EGTA, 9 mM HEPES, 0.4 mM CaCl₂, 14 mM creatine phosphate (Tris salt), 4 mM Mg-ATP, and 0.3 mM GTP (Tris salt), pH 7.4 with KOH.] (Nashmi et al., 2003; Fonck et al., 2005). Also used in Drenan, MolPharm-2008.

Extracellular Liquid-ECL (or Solution-ECS or Fluid-ECF)

After Clemens, Sodickson DL and Bean BP (1998) J. Neurosci. **18**, 8153-8162.

	GMW	mM	X1 l(g)	10X l(g)
NaCl	58.44	150	8.766	87.66
KCl	74.55	4	0.298	2.98
Hepes	238.3	10	2.383	23.83
MgCl ₂	203.3	2	0.407	4.07
CaCl ₂	110.99	2	0.222	2.22
Glucose	180.16	10	1.802	18.02

Adjust to pH 7.4 with NaOH

Coverslip cleaning summary

This is for starting with dirty coverslips and going all the way.

To clean new coverslips, that do not need a specific surface charge, you can simply sonicate in spectroscopic grade acetone and then blow dry.

My standard Glass Cleaning Procedure

June 8, 2010

This is a variation of the RCA method

In the "RCA" method, coverslips and slides were:

1. sonicated in a surfactant solution (2% Micro-90) for 20 min,
2. washed for 3min in a stream of deionized water,
3. rinsed thoroughly with 18.3 M Ω -cm and 0.22 μ m filtered water,
4. then immersed in boiling RCA solution (3:2:1 high-purity H₂O, 30% NH₄OH, 30% H₂O₂) for 20 min.
 - a. Temperature was 78C and the fluid was boiling.
 - b. e.g. for 420 ml of RCA solution (SC-1) combine 210 ml of water with 140 ml of NH₄OH with 70 ml of H₂O₂.
5. Coverslips were rinsed and stored in high purity water to avoid particulate contamination. High-purity water was 18.3 M Ω -cm and 0.22 μ m filtered.
6. Rinses: rinsed 6 times, vigorously in 18.3 M Ω -cm and 0.22 μ m filtered water, filled to brim and vigorously agitated.
7. Immerse in boiling acid solution (SC-2): combine (14:3:1 ratio) 350 ml of water with 75 ml of H₂O₂ with 25 ml of HCl (37%) for 450 ml solution for 10 minutes.
 - a. Remember to add acid to water.
8. Rinses: rinsed 6 times, vigorously in 18.3 M Ω -cm and 0.22 μ m filtered water, filled to brim and vigorously agitated.
9. Store in the same crystallization dishes filled with a 7th round of 18.3 M Ω -cm and 0.22 μ m filtered water.
10. Immediately before use, coverslips were blown dry with filtered nitrogen (MMCFA02 filter: 0.01 μ m filtered, oil removed to 1 part per trillion of line input; Airmaze, Stow, OH, USA).

Alternate methods:

The one step RCA method is probably less aggressive to the glass but it can leave organic stuff behind. All of the methods described below leave the coverslip highly hydrophobic.

It may therefore be necessary to coat with poly-DL-lysine afterward for cells to happily bind to the glass surface.

1. RCA cleaning is the industry standard for removing contaminants from wafers. Werner Kern developed the basic procedure in 1965 while working for RCA (Radio Corporation of America) - hence the name.

In the “RCA” method, coverslips and slides were:

1. sonicated in a surfactant solution (2% Micro-90) for 20 min,
2. washed for 3min in a stream of deionized water,
3. rinsed thoroughly with 18.3 MΩ-cm and 0.22μm filtered water,
4. then immersed in boiling RCA solution (6:4:1 high-purity H₂O, 30% NH₄OH, 30% H₂O₂) for 1 h.
 - a. Temperature was 78C and the fluid was boiling.
 - b. e.g. for 440 ml of RCA solution combine 240 ml of water with 160 ml of NH₄OH with 40 ml of H₂O₂.
5. Coverslips were rinsed and stored in high purity water to avoid particulate contamination. High-purity water was 18.3 MΩ-cm and 0.22μm filtered.
6. Rinses: rinsed 6 times, vigorously in 18.3 MΩ-cm and 0.22μm filtered water, filled to brim and vigorously agitated.
7. Store in the same crystallization dishes filled with a 7th round of 18.3 MΩ-cm and 0.22μm filtered water.
8. Immediately before use, coverslips were blown dry with filtered nitrogen (MMCFA02 filter: 0.01 μm filtered, oil removed to 1 part per trillion of line input; Airmaze, Stow, OH, USA).

An updated version of RCA (from: <http://fabweb.ece.uiuc.edu/recipe/rca.aspx>) recommends:

1. starting with Acetone,
2. DI water rinse
3. Put in an acid rinse: 50:1 H₂O, HF (hydrofluoric acid) for 30 seconds.
4. DI water rinse
5. Then use RCA solution. Their solution uses the same components but in different ratios: 10 (H₂O), 2 (H₂O₂), 1 (NH₄OH). They also use a temp of 75 C. They leave their wafers (coverslips for me) there for ~20 minutes (~10 minute warmup and 10 minutes at 75 C). While this is going on, prepare the next solution.
6. Rinse with DI water
7. Then use a second solution in the ratio (17 (H₂O), 3 (H₂O₂), 1 (H₂SO₄-sulfuric acid)). Remember the AAA rule: **Always add acid to water!** Sulfuric acid reacts violently with water: add it very slowly, and only if the temperature of the water is below 30°C. Put wafers in this solution and then heat to 75 C. Then remove wafer (so time to 75C is ~10 min?).
8. Then do another 50:1 DI:SF etch for 15 seconds (strips hydrous oxides)
9. Rinse in DI water
- 10 Place wafers back into the 17:3:1 H₂O:H₂O₂:H₂SO₄ solution (now heated to 75C). Leave it there for 10 minutes.
11. Rinse in DI water
12. Blow dry with Nitrogen gas.

Third variation: The RCA clean first uses an $\text{H}_2\text{O}-\text{NH}_4\text{OH}-\text{H}_2\text{O}_2$ solution (standard clean 1; SC1) to remove organic contaminants and particles. After rinsing with dilute HF, a second standard solution (SC2) uses a $\text{H}_2\text{O}-\text{HCl}-\text{H}_2\text{O}_2$ mixture to remove metal contaminants. A final rinse prepares the wafer for further processing.

Fourth variation is copied from Wikipedia: http://en.wikipedia.org/wiki/RCA_clean

The RCA clean is a standard set of wafer cleaning steps which needs to be performed before high temp processing steps (oxidation, diffusion, CVD) of silicon wafers in semiconductor manufacturing. RCA cleaning includes RCA-1 and RCA-2 cleaning procedures. RCA-1 involves removal of organic contaminants, while RCA-2 involves removal of metallic contaminants.

Werner Kern developed the basic procedure in 1965 while working for RCA, the Radio Corporation of America^[1] It involves the following :

1. Removal of the organic contaminants (Organic Clean)
2. Removal of thin oxide layer (Oxide Strip)
3. Removal of ionic contamination (Ionic Clean)

The wafers are prepared by soaking them in DI water. The first step (called SC-1, where SC stands for Standard Clean) is performed with a 1:1:5 solution of NH_4OH (ammonium hydroxide) + H_2O_2 (hydrogen peroxide) + H_2O (water) at 75 or 80 °C^[1] typically for 10 minutes. This treatment results in the formation of a thin silicon dioxide layer (about 10 Angstrom) on the silicon surface, along with a certain degree of metallic contamination (notably Iron) that shall be removed in subsequent steps. This is followed by transferring the wafers into a DI water bath.

The second step is a short immersion in a 1:50 solution of $\text{HF} + \text{H}_2\text{O}$ at 25 °C, in order to remove the thin oxide layer and some fraction of ionic contaminants.

The third and last step (called SC-2) is performed with a 1:1:6 solution of $\text{HCl} + \text{H}_2\text{O}_2 + \text{H}_2\text{O}$ at 75 or 80 °C. This treatment effectively removes the remaining traces of metallic (ionic) contaminants^[1].

The RCA cleaning procedures leaves hydroxyl groups on the glass surface, which is deprotonated at the pH used here, and thus impart negative charge to the surface (making it hydrophilic). While this charge can provide some electrostatic shielding against non-specific adsorption of tagged nucleotides, the surface charge density is very low.

Notes:

H_2O_2 is hydrogen peroxide

NH_4OH is ammonia hydroxide

A more aggressive method:

- 1) Fill a clean teflon rack with coverslips (VWR, No.1, 1" square) and place in a clean crystallization dish.
- 2) Cover the glass coverslips with Micro-90 cleaning solution. Sonicate for 30 min and rinse in DI water.
- 3) Fill the crystallization dish with 30 % (w/w) NaOH. Sonicate the totally immersed coverslips at room temperature for 1 h.
- 4) The coverslips must be rinsed in DI water again, until they reach pH = 6.5 (pH of our laboratory water).
- 5) Steps 3) and 4) are repeated with 1 N HCl.
- 6) Refill and store coverslips in 18 M Ω water

Cell Lysis

AfCS Solution Protocol

Reagent name: Hypotonic cell lysis buffer stock, pH 7.4, 2X

Reagent name abbreviation: 2X HSE, pH 7.4

Protocol ID: PS00000678

Version: 01

Volume: 250 ml

Components: Reagent	Source	Catalog or Protocol No.	F.W. or Stock Conc.	Quantity	Final Concentration
HEPES	Invitrogen	15630080	1 M	10 ml	40 mM
Sodium chloride (NaCl)	None	PS00000677	4 M	2.5 ml	40 mM
EDTA, pH 8.0	None	PS00000026	0.5 M	5 ml	10 mM
Hydrochloric acid (HCl)	None	PS00000034	1 N	titrate	NA
Sodium hydroxide (NaOH)	None	PS00000038	1 N	titrate	NA

Preparation:

1. Pipette HEPES, NaCl, and EDTA in order into a 250-ml beaker.
2. Add purified water to a volume of approximately 220 ml.
3. Adjust pH to 7.4 with 1 N HCl or 1 N NaOH.
4. Adjust volume to 250 ml with purified water in a graduated cylinder.
5. Sterilize by autoclaving or filtration and divide into 50-ml aliquots.

Storage:

Temperature: 4 °C

Aliquot size: 50 ml

Author: Leah Santat

Date: 09/01/04

Approved: Iain Fraser

Membrane patch preparation:

Adhere the cell onto an APTES coated glass coverslip. Plate cells on coverslip and let grow.

Prepare coverslips using the Pierce protocol.

Wash with ice cold PBS once to remove loose cells

Pour into edge of dish and let sit for 1 min., then pour out

Lyse the cell:

Low concentration ionic buffer solution: 18 MΩ water with 5 mM NaH₂PO₄ and 1 mM EDTA (pH 7.1). **Do this in the refrigerator.** Let sit for 12.5 minutes.

Cell lysis (splitting of cells) is when a cell gets so fat from all the hypertonicity that it just explodes. Cytolysis is the lysis of cells in a hypotonic environment. Cytolysis is caused by excessive osmosis, or movement of water, towards the inside of a cell (hyperhydration). The cell membrane cannot withstand the osmotic pressure of the water inside, and so it explodes. Osmosis occurs from a region of high water potential to a region of low water potential passing through a semipermeable membrane, so these bursting cells are located in hypotonic environments. (from wikipedia). EDTA chelates Ca ions involved in intercellular and intra-cellular adhesion and results in better breakdown of cells.

Wash the cells to remove everything but the adhered membranes. **Do this with ice cold solution.**

Wash in ice cold PBS.

Again **pour into edge of dish, let sit for 1 min. then pour out**

Replace with PBS and then look under microscope to make sure the patches look good.

Try imaging membrane patches with the membrane skeletal structure intact in PBS.

Dissolve the membrane skeletal structure

Remove the filamentous actin/spectrin mesh by incubation with a 0.5 mM Na phosphate buffer (0.5 mM NaH₂PO₄, 0.05 mM EDTA, pH 7.4) at 37 °C for 30 min.

Place cells in incubator in hallway.

Gently wash again with PBS.

Image wet in PBS.

Mixing Protocols:

Lysing solution: 5 mM NaH₂PO₄ and 1 mM EDTA (pH 7.1)

Make about ½ liter.

Start with 500 ml of 18 MΩ water in a flask

½ liter x 0.005 M x 137.99 g/Mol=0.345 g of NaH₂PO₄ H₂O

Add 0.345 g of NaH₂PO₄ H₂O

We have 0.5 M EDTA. And want 1mM (500 times more dilute).

Add 1 ml EDTA for 500 ml.

Mix using a stirring bar and measure pH. Add NaOH until at pH=7.1.

Then vacuum feed and filter into 500 ml container.

Membrane skeletal structure removal: 0.5 mM NaH₂PO₄, 0.05 mM EDTA, pH 7.4

Make about ½ liter.

Start with 500 ml of 18 MΩ water in a flask

½ liter x 0.0005 M x 137.99 g/Mol=0.0345 g of NaH₂PO₄ H₂O

Add 0.0345 g of NaH₂PO₄ H₂O

We have 0.5 M EDTA. And want 0.05mM (10000 times more dilute).

Add 50 µl EDTA for 500 ml.

Mix using a stirring bar and measure pH. Add NaOH until at pH=7.4.

Then vacuum feed and filter into 500 ml container.

N2a cell top membrane adhesion to coverslip

February 6, 2009

Use an APTES coated coverslip

Prepare using Pierce instructions and product #80370:

Protocol for Amino-Silylation of a Glass Surface

1. Thoroughly wash and dry the glass, silica or quartz surface to be coated.

Note: Perform steps 2 and 3 in a fume hood.

2. Prepare a 2% solution of 3-Aminopropyltriethoxysilane in acetone. For example, mix 1 part Amino-silane Reagent with 49 parts dry (i.e., water-free) acetone. Prepare a volume sufficient to immerse or cover the surface material.

3. Immerse surface in the diluted reagent for 30 seconds.

4. Rinse surface with acetone.

5. Allow surface to air-dry.

Note: The dried silylated surface may be stored for later use.

Plate and transfect N2a cells as normal in Matek dish with 30 mm hole/coverslip

Wash out N2a cell media and replace with ECL

Place APTES coated 25mm round coverslip on top of cells

First try laying gently on top...if no adhesion try giving a very gentle push on each corner to ensure cell contact

Incubate cells for 30 min at 37 C to give time for adhesion to occur

Lift up coverslip (tearing cells) and place in dish

Wash both coverslips (with cell bottom and top membranes respectively) with intracellular fluid

Image the coverslip with the adhered membranes isolated from the cell top will need to be imaged 'open' (not in a dish) under a meniscus of intracellular fluid.

Linking carboxylated beads to APTES coated coverslip

EDAC is a water-soluble carbodiimide

EDC (also EDAC or EDCI, acronyms for 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide)

[http://en.wikipedia.org/wiki/1-Ethyl-3-\(3-dimethylaminopropyl\)carbodiimide](http://en.wikipedia.org/wiki/1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide)

<http://en.wikipedia.org/wiki/Carbodiimide>

MES is [http://en.wikipedia.org/wiki/MES_\(buffer\)](http://en.wikipedia.org/wiki/MES_(buffer))

2-(N-morpholino)ethanesulfonic acid is MES buffer

The MES I bought makes 0.1M at 4.7 pH. Dilute with DI water to 50 mM. Then titrate with NaOH to pH 6.0 (maybe 6.5 TBD).

To link carboxylated beads to amine groups (APTES coated glass)

Some variant: 50 mM MES at pH 6, add microspheres. Then add 4 mg fresh EDAC to a 1 ml MES/Bead mixture. Let incubate on amine (APTES) coated slide for 15 minutes at room temperature. If want to avoid non-specific binding at this point passivate with dilute BSA.

The MES causes the carboxyl groups to be exposed and active. Otherwise they tend to be shielded due to charge. However the beads should not be in an environment < 5 pH. pH 6.0 is probably optimal and stay in the range of $5.5 < \text{pH} < 6.5$.

APTES coating glass coverslips

Silanization procedure:

- 1) First the described cleaning procedure must be applied to the coverslips.
- 2) The surfaces are placed in a polymethylpentene (PMP) jar with 0.6 mM APTES solution in 100 % ethanol (for APTES see Sigma Aldrich catalog).
- 3) The coverslips are stored in this solution for 48 h.
- 4) After drying the coverslips should be used for imaging during the next few hours.