APTES-DSC Protocols

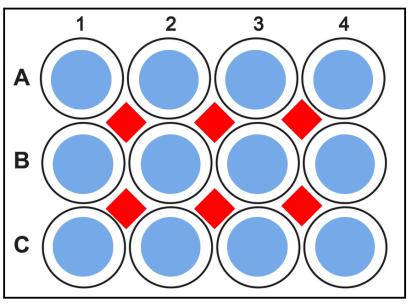
Coverslips

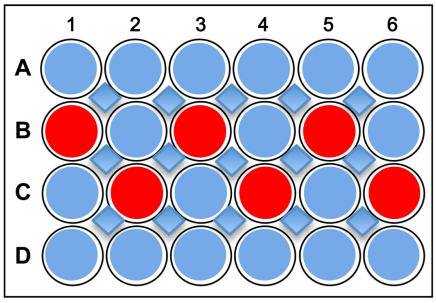
Materials
Glass coverslips (15 mm – 24-well plate or 18 mm – 12-well plate, Fisher)
(UV/ozone cleaner, BioForce Nanosciences)
(3-aminopropyl)triethoxysilane (APTES, Sigma)
Toluene
95% Ethanol
Water (nanopure or equivalent)
N,N-disuccinimidyl carbonate (DSC, Sigma)
Diisopropylethylamine (DIEA, Sigma)
Acetone
Protein/peptide solution in sterile PBS, pH 7.4
Tweezers
MA-PEG_{24} (VWR)
Devcon 5 Minute Epoxy (Fisher or RS Hughes)

Day 1 (20min, longer for more samples)

1. UV/ozone or plasma treat glass coverslips for 10 minutes. Stand coverslips up in a well plate so both sides are exposed to air. i.e., two 18 mm coverslips can be placed to make a V in a well of a 12-well plate. If you use 15 mm coverslips, use a 24-well plate.
    a. This is important because you will not be able to keep track of directionality! Do not use any coverslips that have fallen over.
2. Add 75 uL of APTES to six places in the well plate, but in places that your coverslips are not. Do not put silane directly onto coverslips, this is a vapor deposition reaction.
    a. 12-well plate: I use the 6 spaces in between wells (spaced throughout the plate, blue = coverslips, red = silane)
    b. 24-well plate: I keep 6 wells empty evenly spaced throughout the plate (33 coverslips per plate, blue = coverslips, red = silane)

    Using plastic tissue culture plates is not ideal for the vapor deposition and it would be better to do this step in a glass container.





3. Put lid on plate, seal around edges with autoclave tape to contain silane, being careful not to knock over any coverslips. Wrap plate in aluminum foil to contain silane again, label with autoclave tape.
4. Put in oven at 85-90°C overnight (12-18 h minimum).
    a. Too hot: At 90°C or above, the well plate will warp and coverslips will get stuck and break when you try to take them out! If this is an issue, decrease temperature slightly. Also be aware of where the heating element is in the oven – ours is on the bottom, so I put my plates with coverslips on the top rack to decrease heating issue.

Day 2 (3.5-4 hours)

1. Take coverslips out of oven and rinse each coverslips 4x in toluene, 4x in 95% ethanol, and 4x in water with tweezers by dipping.
    a. I make 50 mL tubes of each solvent and use tweezers to dip one coverslip in toluene, ethanol, and then water, then place to dry on foil.
        i. Drying: take a piece of foil and fold ridges into it so coverslips can be stood up to dry more quickly and evenly
2. After all coverslips have been rinsed this way, place foil containing coverslips back into the oven at 85-90°C to dry (1 h minimum)
3. Place coverslips into a solution of 10 g/L DSC, 5 v/v% DIEA in acetone and shake at room temperature for 2 h. 30 mL is sufficient for 24 18 mm coverslips in a clean, dry beaker.
    a. I tape the beaker onto shaker at ~115 rpm, just fast enough that coverslips move around in beaker. Larger beakers allow for better movement of coverslips.
    b. Alternative: use a stir bar and stir plate to move solution and coverslips, but be careful that it's not so fast that it breaks them. This method is not preferred.
4. Rinse coverslips individually 3x in acetone and dry on folded foil tray in air.

5. When dry, ~10 min, fold up foil and store in dessicator for 1 day, or use immediately.

Day 3 (or continuation of day 2; 6+ hours)

1. Make protein solution in sterile PBS so that desired surface concentration of proteins is in 95 uL (18 mm) or 70 uL (15 mm). This can be done on the bench if UV-sterilization is available, or in the tissue culture hood. Sterile PBS is key to keeping these clean!

Concentrations between 1-10 μm/cm^2 have been used in the lab.
Note: For collagen I, concentrations between 1-5 μg /cm^2 use sterile PBS, pH 3.8

The table below is description for a 18 mm coverslip and 1 μg/cm^2.

$$Area = \pi r^2$$
$$Area = \pi r(0.9\ cm)^2 = 2.54\ cm^2$$

Mass need per 1 slide

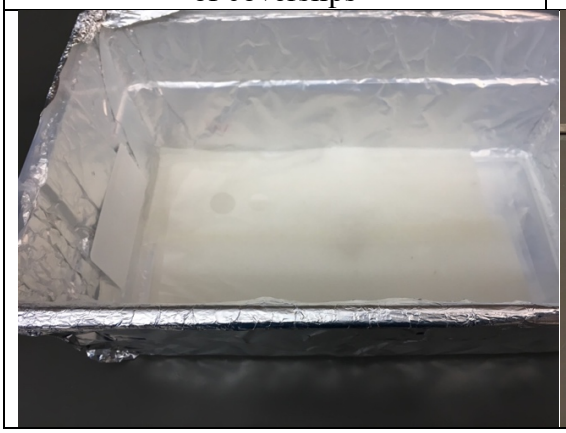
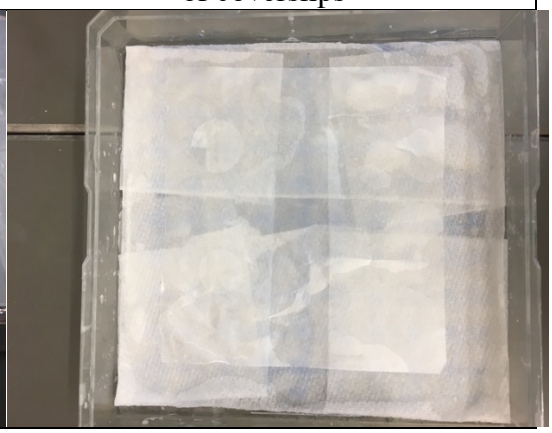
$$1\frac{\mu g}{cm^2} * 2.54\ cm^2 = \ 2.54\ \mu g$$

Volume needed of protein per 1 slide

Protein	Concentration	Volume needed of protein per 1 slide
Collagen	3 ug/uL	2.54 ug/ 3 ug/uL = 0.846 uL
Laminin	1.19 ug/uL	2.54 ug/ 1.19 ug/uL = 2.13 uL
Fibronectin	1 ug/uL	2.54 ug/ 1 ug/uL = 2.54 uL

Complete volume with PBS ➔ 2.13 μl + 92.87 μl = 95 μL

2. Use a container large enough to hold all coverslips and line the bottom with damp paper towels. The water in the paper towels and keeps coverslips hydrated.
3. Add Parafilm into the container, remove the paper, and add 95 uL (or 70 uL) droplets of protein solution spaced out on the Parafilm. They should stay as droplets because of hydrophobicity, but do not space them too close because then your coverslips might touch.

Set up if you are running large quantity of coverslips	Set up if you are running small quantity of coverslips
	

4. Carefully use tweezers to place a coverslip on each protein solution droplet. Use the tweezers to carefully move the coverslip if the whole surface is not wetted or there is an air bubble.
5. Incubate for 3 hours. **Now the coverslips have directionality! Protein side is down.**
6. Move coverslips into a well plate, flipping the orientation (protein side up), and rinse wells 4x in sterile PBS. Again, better in the tissue culture hood but ok on the bench with sterile PBS.
   a. 18 mm coverslips can be rinsed in a 12-well plate and removed
   b. 15 mm coverslips cannot be removed once placed into 24-well plate, so rinse in 12-well plate.
7. Replace the Parafilm and add new droplets of the blocking solution.
   Blocking solution (10 μg/cm^2 MA-PEG$_{24}$ in PBS)

   For an 18 mm coverslip, we have $2.54\ cm^2$

   Mass need per 1 slide

$$10\,\frac{\mu g}{cm^2} * 2.54\ cm^2 = \ 25.4\ \mu g$$

   Volume needed of protein per 1 slide (stock solution 5 μg/μl)

   25.4 μg/ 5 μg/μl = 5.08 μl

   Complete volume with PBS ➔ 5.08 μl + 92.87 μl = 89.92 μL

8. Incubate for 2 hours.
9. Move the coverslips back into the well plate, rinse 4x in sterile PBS.
10. Epoxy the coverslips to the well plate if you will be doing time lapse microscopy.
    a. Mix a little bit of each of the 2 components of the epoxy on some foil with a pipet tip

b. 18 mm: use tweezers to lift coverslip, add a small dab of epoxy to bottom, and place coverslip back in well. Push down with tweezers to stick it to the bottom. It should cure in 5 min, even in PBS.
c. 15 mm: add epoxy BEFORE putting coverslip into 24-well plate. Wait 5 min before rinsing because this will agitate epoxy all over the coverslip.
    i. Application of epoxy can either be done with a pipet tip or 1 uL using a positive displacement pipet. Do not add too much because it is difficult to image through.

11. Either use immediately, or seal plate with Parafilm and store at 4°C for 1 day. UV-sterilization for 30-60 min minimum is preferred, but not necessary, for short-term experiments.
    a. If contamination problems, do all in the hood, UV sterilize, and make sure PBS is filtered/autoclaved.

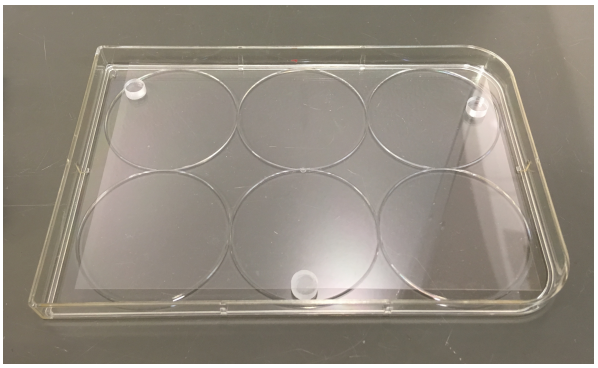
APTES-DSC 96-well Plates

Materials

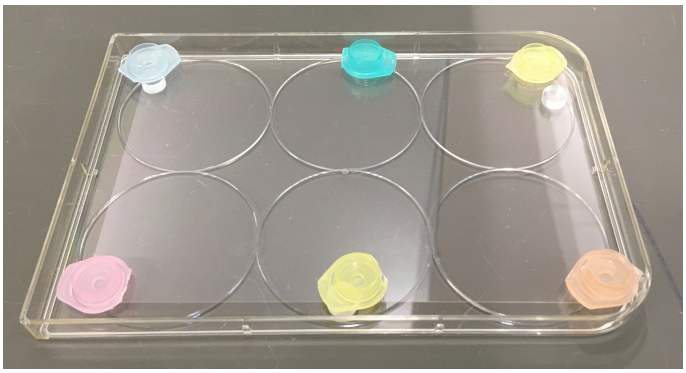
Glass (75 mm by 110 mm, 0.15 mm thick (same as number 1.5 coverslip), Howard Glass, Worcester, MA)
UV/ozone cleaner (BioForce Nanosciences)
2 well plate lids (make sure they are Falcon because other brands don't seem to hold up in the oven)
Six 1.5 mL microcentrifuge tube lids
(3-aminopropyl)triethoxysilane (APTES, Sigma)
Toluene
95% Ethanol
Water (nanopure or equivalent)
N,N-disuccinimidyl carbonate (DSC, Sigma)
Diisopropylethylamine (DIEA, Sigma)
Acetone
ECM proteins
PBS
Bottomless 96-well plate (Greiner Bio-One 655000-06) with ARcare 90106 double-sided adhesive (Adhesives Research)
MA-PEG_{24} (VWR)

Day 1 (20min, longer for more samples)

1. UV/ozone or plasma treat the glass for 10 minutes. Place the glass in a well plate lid on small acrylic discs (about the diameter of one well in a 96-well plate) to allow for air flow on both sides of the glass.



2. Add 75 uL of APTES to six inverted 1.5 mL microcentrifuge tube lids evenly spaced around the edges of the glass. Do not put silane directly onto the glass, this is a vapor deposition reaction.



3. Put another lid on the lid with the glass, seal around edges with autoclave tape to contain silane, being careful not to move lids with the APTES. Wrap plate in aluminum foil to contain silane again, label with autoclave tape. It would be better to do this in a glass container rather than plastic, if possible.
4. Put in oven at 85-90°C overnight (12-18 h minimum).
    a. Too hot: At 90°C or above, the well plate lids will warp and it may be difficult to get the glass out. If this is an issue, decrease temperature slightly. Also, be aware of where the heating element is in the oven – ours is on the bottom, so I put my plates on the top rack to decrease heating issue.

Day 2 (3.5-4 hours)

1. Take the glass out of oven and rinse 4x in toluene, 4x in 95% ethanol, and 4x in water with tweezers by dipping into basins with each solvent.
    a. I put each solvent in a basin that is large enough to hold the glass (make sure the materials are compatible with the solvent) and use tweezers to dip each piece of glass in toluene, ethanol, and then water, then place to dry on foil.
        i. Drying: take a piece of foil and fold ridges into it so there is air flow on both sides of the glass to help the glass to dry more quickly and evenly.
2. Place the foil containing the glass back into the oven at 85-90°C to dry (1h minimum)
3. Place the glass into a solution of 10 g/L DSC, 5 v/v% DIEA in acetone and shake at room temperature for 2h. 30 mL is sufficient for one piece of glass in an inverted positive displacement tip box, sealed around the edges with Parafilm.
    a. I tape the box onto shaker at ~115rpm, just fast enough that glass moves around in the box.

4. Rinse the glass 3x in acetone in a basin and dry on folded foil tray in air. When dry, ~10 min, fold up foil and store in desiccator for 1 day, or use immediately.

Day 3 (or continuation of day 2; 6+ hours)

1. Make protein solution in sterile PBS so that desired surface concentration of proteins is in 45 uL/well. This can be done on the bench if UV-sterilization is available, or in the tissue culture hood. Sterile PBS is key to keeping these clean! (See additional spreadsheet for quick calculations and combinations).

Protein	Concentration	Volume needed of protein per 1 well
Collagen	3 ug/uL	0.34 ug/ 3 ug/uL = 0.113 uL
Laminin	1.19 ug/uL	0.34 ug/ 1.19 ug/uL = 0.286 uL
Fibronectin	1 ug/uL	0.34 ug/ 1 ug/uL = 0.34 uL

2. Attach the silane treated glass to a bottomless 96-well plate (Separate protocol to prepare this).
3. Add 45 uL droplets of protein solution to each well. Make sure the solution covers the entire well surface. Only use the interior 60 wells of the plate due to evaporation. Fill the outer wells with 100 uL PBS in each well.
4. Incubate for 3 hours on the bench covered with aluminum foil.
5. Rinse wells 4x with 100 uL/well sterile PBS. Again, better in the tissue culture hood, but ok on the bench with sterile PBS.
6. Add 45 uL of the blocking solution (10 ug/cm^2 PEG_{24} in PBS) to each well.
7. Incubate for 2 hours on the bench covered with aluminum foil.
8. Rinse 4x with 100 uL/well sterile PBS.
9. Either use immediately, or seal plate with Parafilm and store at 4°C for 1 day. UV-sterilization for 30-60 min minimum is preferred, but not necessary, for short-term experiments.
   If there are contamination problems, do all in the hood, UV sterilize, and make sure PBS is filtered/autoclaved