Biological Sample Preparation

331.1 Introduction

Atomic force microscopy (AFM) provides a means to image many biological structures. A number of techniques have been developed to preserve samples through the imaging process and to optimize sample presentation for imaging. Some of these techniques are described here in the following sections:

Document Revision History: Biological Sample Preparation

Revision	Date	Section(s) Affected	Reference	Approval
A	8-Oct-01	Initial Release	435	Alan Rice

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331.2 General Notes On Sample Binding

Samples for atomic force microscopy imaging should be immobilized on a rigid support. Biological specimens like cells, molecules (proteins, DNA, etc.) and Langmuir-Blodgett films are usually bound to a flat substrate like mica or glass. Mica is a very common support because the surface is:

- atomically flat
- clean after cleavage
- easy to cut to desired sizes
- relatively inexpensive
- negatively charged—but it can be modified to make the surface positive.

Use the following basic recipes as guides to imaging your samples. Specimen binding is usually accomplished using electrostatic attraction between charges on the specimen and charges on the mica surface. Proteins, for example, exhibit positive charges, especially when the buffer pH is lower than the isoelectric pH of the proteins. DNA, on the other hand, is negatively charged and can be bound either by creating positive charges at the surface (using a silanization process, see below), or by dissolving DNA in a divalent metal counter ion (e.g. Mg^{2+}, Ni^{2+}). Both of these techniques are discussed separately below.

Sample preparation remains a challenging part of AFM studies in biology. It is still challenging to immobilize red blood cells or bacteria, for examples, to make them stick on a surface after immersion in a buffer solution. Tissues are also difficult to study for that reason, and also because of their softness. However, the steady progress in immobilization techniques explains the improved results being obtained daily. Keep in mind that it can take a while to find the right conditions to prepare, and image, your sample.

331.3 DNA Molecules

DNA is typically prepared by incubating it on mica. Since both DNA and mica are negatively charged, divalent cations, such as Ni^{2+} or Mg^{2+}, are added to the buffer to bridge the negative charges of DNA and mica. As an alternative, DNA can be prepared in Tris-EDTA buffer, without cations, and incubated on AP-mica (silanized mica—see recipe below). TappingMode™ (in either air or fluid) is the preferred imaging technique. In Contact Mode the tip dislodges DNA molecules from the substrate.

331.3.1 DNA Binding Theory

Because DNA and mica are both negatively charged, it is necessary to modify the mica surface or the DNA counter ion to allow binding. The counter ion method is performed by adsorbing DNA onto the mica in the presence of cations, like Ni^{+2}. The divalent ion serves as a counter ion on the negatively charged DNA backbone and also provides additional charge to bind the mica.

331.3.2 DNA Binding Procedure

To prepare a DNA sample, 50µl of 1-10µg/ml of DNA (commercial step ladder, plasmid, etc.) is deposited onto freshly cleaved mica (already glued on a steel disc if necessary) and incubated for 5-10 minutes. For imaging in air, the mica is rinsed with buffer and dried with nitrogen (or compressed air). For fluid imaging, the DNA sample is ready to go—rinsing is optional. A typical buffer to dilute the stock DNA solution contains 40mM HEPES-HCl, 10mM $NiCl_2$, at pH 6-7.4. Silicon cantilevers (TESP) are used for imaging in air whereas silicon nitride probes (DNP-S, NP-S or OTR-4) are used for imaging in liquid. We recommend short, narrow (100µm long) V-shaped cantilevers.

331.3.3 References

1. Hansma, H.G. and Laney, D.E. (1996), “DNA binding to mica correlates with cationic radius: assay by atomic force microscopy,” Biophys. J. 70: 1933-1939.

2. Mou, J., Czajkowsky, D. M., Zhang, Y. and Shao, Z. (1995), “High-resolution atomic-force microscopy of DNA: the pitch of the double helix,” FEBS Lett. 371(3): 279-282.

3. Dunlap, D.D., Maggi, A., Soria, M.R. and Monaco, L. (1997), “Nanoscopic Structure of DNA Condensed for Gene Delivery,” Nucl. Acids Res. 25, 3095.

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331.4 Proteins

Proteins can be imaged in both air and fluid using either Contact Mode or TappingMode. If imaging in air, a contamination layer may prevent you from visualizing protein molecules. For fluid imaging, the pH of the buffer should be lower than the isoelectric point (pI) of the protein because proteins are positively charged at pH below their isoelectric point. (All proteins contain free amino groups that become positively charged at relatively low pH.) If sufficient free amino groups are located on the outside surface of the protein, then the protein will bind to the negatively charged mica surface.

331.4.1 Sample Preparation

Sample preparation varies slightly depending on the protein itself. Typically, protein concentration ranges from 1-100µg/ml. The incubation time also varies from as little as 15 minutes to up to 24 hours. After the proper incubation period, rinse the sample with fresh buffer. For best results, use the narrow silicon nitride tips for TappingMode AFM. It may also be necessary to adjust the electrolyte solution and pH of the buffer in order to minimize the applied forces on the sample.

331.4.2 Lysozyme – an Example

This section gives a detailed procedure for preparing and imaging the protein lysozyme by TappingMode in fluid. The procedure was kindly provided by Monika Fritz at U. Goettingen, Germany, and is described in the following paper: Radmacher, M., Fritz, M., Hansma, H.G., Hansma, P.K. (1994), “Direct Observation of Enzyme Activity with Atomic Force Microscopy,” Science 265, 1577.

The protein lysozyme becomes sufficiently positively charged to bind to mica at a pH of 6.

Required Materials

- De-ionized water
- Mica substrates
- Lysozyme protein (from Sigma Chemical, for example)
- Phosphate buffer solution, 10mM KH_2PO_4, 150mM KCl, pH 6 (the buffer may be adjusted for other proteins)
- TappingMode Fluid Cell
- Cantilevers (oxide-sharpened silicon nitride tips, Model DNP-S, work well)
- Source of filtered (0.2µm), compressed air or dry nitrogen
- Micropipettes

- Optional for cantilever cleaning: UV lamp, high intensity; Oriel Mod. 6035 pencil-style spectral calibration lamp or equivalent.
- Optional: Fluid cell liquid lines (silicone tubing and fittings), o-ring, clamping devices (for liquid lines), syringes: (1) 1cc; (2) 5cc.

Protocol

1. Dissolve protein

Dissolve the lysozyme in phosphate buffer (PBS) to a concentration of 1mg/ml. This concentration provides convenient coverage for AFM imaging and may be used for a variety of similar size samples.

2. Prepare fluid cell

Prepare the fluid cell for TappingMode in fluid operation. Clean the fluid cell and load a probe. For best results, clean the probe with UV light.

3. Prepare mica

Cleave a fresh mica surface by first pressing some adhesive tape against the top mica surface, then peeling off the tape. Glue mica to a small puck (e.g., using epoxy).

4. Deposit protein solution on mica

Deposit 50µl of protein solution on the freshly cleaved mica.

5. Allow protein to bind to substrate

Allow 20-30 minutes for the protein solution to bind to the mica substrate. Binding time may vary with different samples (it can be up to 24 hours). For longer binding times, put the mica in a covered dish with a wet piece of filter paper to keep the liquid from evaporating.

6. Rinse unbound proteins from fluid cell

Rinse the sample with a large quantity of buffer to remove unbound protein. Leave a drop of buffer on the mica.

7. Assemble fluid cell

Mount the sample on the scanner end cap.

Note: It is also possible to prepare samples inside the fluid cell by flowing the protein solution through the fluid cell. In this case, it may be helpful to engage the tip in Contact Mode with a zero **SCAN SIZE** to protect proteins from binding to the tip.

331.4.3 References

1. Hallett, P., Tskhovrebova, L., Trinick, J., Offer, G. and Miles, M. J. (1996), "Improvements in atomic force microscopy protocols for imaging fibrous proteins," J. Vac. Sci. Technol. B. 14(2): 1444-1448.

2. Möller, C., Allen, M., Elings, V., Engel, A. and Müller, D. J. (1999), "Tapping-mode atomic microscopy produces faithful high-resolution images of protein surfaces," Biophys. J. 77: 1150-1158.

3. Müller, D. J., Fotiadis, D., Scheurig, S., Müller, S. A. and Engel, A. (1999), "Electrostatically balanced subnanometer imaging of biological specimens by atomic force microscopy," Biophys. J. 76(2): 1101-1111.

331.5 Living Cells

The BioScope AFM is the instrument of choice. It has been designed especially for cell imaging and can be coupled to epifluorescence. Living cells in culture can be studied using both Contact and TappingMode AFM. In Contact Mode, images mainly show the cytoskeleton beneath the membrane. However, with TappingMode, more cellular details may be observed: the membrane, the nucleus, and vesicles beneath/on the membrane.

331.5.1 Sample Preparation

For optimal imaging, cells grown in 60mm petri dishes should be ~70-80 percent confluent. In most cases, no additional preparation is necessary (glutaraldehyde fixation, etc.); cells should attach to the bottom surface of the petri dish, especially if they are adherent cell lines. If cells are not firmly attaching to the petri dish, you may need to use such adhesives as poly-L-lysine, collagen, laminin, entactin, CellTak™, or polyethylene glycol (PEG) derivatives. The narrow, long (200µm length) V-shaped cantilever (DNP) with a nominal spring constant of 0.06N/m is used to minimize applied forces. Use a non-sharpened (or a tipless cantilever) to avoid destruction of the cell membrane. To minimize damage to the cells, select a small RMS free amplitude of ~0.5V (TappingMode) or a small deflection signal of ~-0.5V (Contact Mode). For both imaging techniques we recommend a slow scan rate of 0.25 to 0.50Hz to start.

Figure 331.5a Contact Mode Images of Endothelial Cells

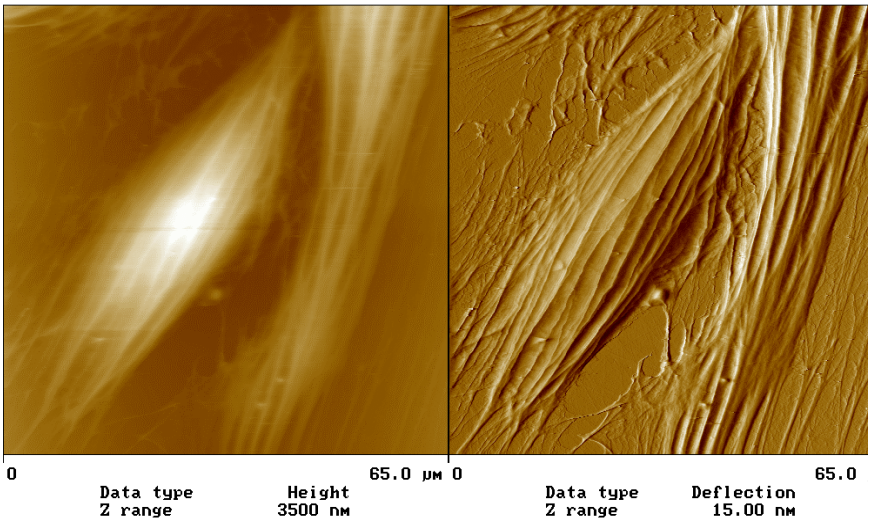
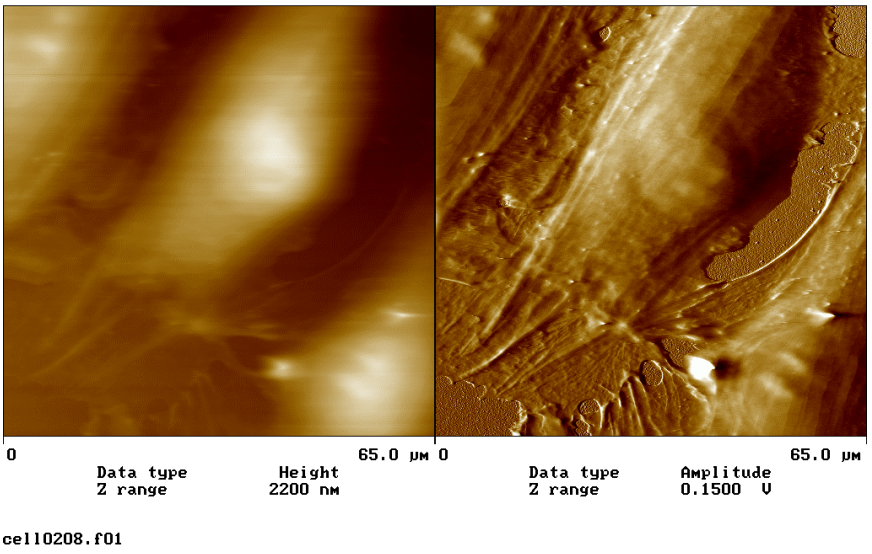


Figure 331.5b TappingMode Images of Endothelial Cells



331.5.2 References

1. Rotsch, C., Jacobson, K.A. and Radmacher, M. (1997), “Investigating living cells with the atomic force microscope,” Scanning Microsc. 11: 1-8.

2. Schilcher, K., Hinterdorfer, P., Gruber, H.J. and Schindler, H. (1997), “A non-invasive method for the tight anchoring of cells for scanning force microscopy,” Cell Biol. Int. 21(11): 769-778.

331.6 Lipids

Two techniques commonly used to attach lipids to bare mica are the Langmuir-Blodgett (LB) technique and the Vesicle Fusion Method. Lipid films usually adsorb spontaneously on mica and can be imaged in air or in liquid, in both Contact Mode and TappingMode, sometimes with molecular resolution.

331.6.1 Langmuir-Boldgett Technique

For the LB technique, replace the usual glass surface used as a support with a piece of mica of the same size. After deposition of the lipid film, the mica can be cut and glued to a sample puck using 5 minute epoxy if necessary. The challenge at this point is to keep the sample under liquid to preserve the molecular structure. This technique is reproducible and enables observing large sheets of organized molecules.

331.6.2 Vesicle Fusion Method

The Vesicle Fusion Method requires that you first obtain a solution of vesicles in buffer. The vesicle solution is first deposited onto mica or another substrate. During the incubation period the vesicles will settle, spread and fuse to the surface at room temperature in a relatively short time (e.g., about 15 minutes for phosphatidylcholine vesicles on mica). Usually the lipid films are not as large as with the LB technique. They form patches on the surface or include ‘defects’, where the substrate is not covered. This protocol is interesting for integrating proteins inside the film to more closely simulate cell membranes and to study lipid/protein interactions.

331.6.3 References

1. Hui, S.W., Viswanathan, R., Zasadzinski, J.A., and Israelachvili, J.N. (1995), “The structure and stability of phospholipid bilayers by atomic force microscopy,” Biophys. J. 68: 171-178.

2. Grandbois, M., Clausen-Schaumann, H., and Gaub, H. (1998), “Atomic force microscope imaging of phospholipid bilayer degradation by phospholipase A2,” Biophys. J. 74: 2398-2404.

3. Egawa, H. and Furusawa, K. (1999), “Liposome adhesion on mica surface studied by atomic force microscopy,” Langmuir 15: 1660-1666.

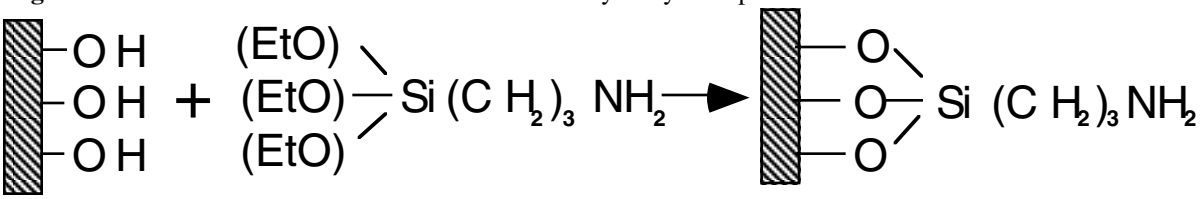
4. Ohler, B., Revenko, I., and Husted, C. (2000), “Atomic Force Microscopy of Non-hydroxy Galactocerebroside Nanotubes and Their Self-assembly at the Air-water Interface, with Applications to Myelin,” J. Struct. Biol.: 133(1): 1-9.

331.7 Silanized Mica (AP-mica): DNA, RNA and Nucleoprotein Complexes Immobilized on AP-mica

By: Yuri L. Lyubchenko, Alexander Gall and Luda Shlyakhtenko (Arizona State University)

We describe here a sample preparation procedure for AFM with the use of functionalized mica substrates. This technique allows routine visualization of DNA, RNA and nucleoprotein complexes with AFM [1-5]. The method is based on covalent bonding of 3-aminopropyltriethoxy silane (APTES) molecules to mica (i.e., creating positive charges at the mica surface) [1,2]. The reaction is shown schematically in Figure 7a.

Figure 331.7a Mica Modification with APTES. The Hydroxy Groups on the Left are Part of the Mica Surface.



The amino groups of APTES are covalently bound to the freshly cleaved mica surface, giving it properties similar to anion exchange resins used in affinity chromatography. The amino groups, after exposure to a water solution, become positively charged in a rather broad range of pH; aliphatic amino-groups have a pK of around 10.5. Therefore, DNA, which is a negatively charged polymer, should adhere strongly to this surface. We checked the DNA binding to AP-mica directly by use of radiolabeled DNA [1]. AFM images of mica substrates modified with APTES in toluene solutions showed that the surfaces are very rough if concentration of APTES is 1μM or more. The surfaces are smooth if APTES concentration in solution is 100nM [1]. The concentration of APTES in solution needs to be so low that mica modification can be performed in APTES vapors at ambient conditions.

331.7.1 Materials

- Commercially available 3-aminopropyltriethoxy silane (e.g. Fluka and Chemika-BioChemika in Switzerland, Aldrich in the USA)

  Note: Ask for a freshly prepared chemical, redistill it and store the chemical under argon to prevent polymerization.

- Mica substrate: any type of commercially available mica sheets (e.g., green or ruby mica).

- Water: double glass distilled or de-ionized water filtered through a 0.5μm filter.

331.7.2 Substrate Preparation

Place two plastic caps—cut them from regular eppendorf tubes—on the bottom of a 2l desiccator and evacuate it with a regular vacuum pump (to -28 inches Hg is sufficient) and fill with Ar. Cleave mica sheets to make them as thin as 0.05-0.1mm. Put 30µl of APTES into one plastic cap and 10µl of N,N-diisopropylethylamine (Aldrich) into another cap. Mount mica strips at the top of the desiccator and leave the reaction to proceed for 1-2 hours. After that, remove the cap with APTES and purge argon for 2 minutes. Leave the sheets to cure for 1-2 days; then AP-mica substrates are ready for the sample deposition.

Note: A dry argon atmosphere is crucial for obtaining substrates for AFM studies and for substrate storage. Allow the gas to flow while you open the desiccator. Under these conditions the AP-mica substrates retain their activity for at least a month.

331.7.3 Sample Preparation

1. **The Droplet Procedure.** Place 50µl of DNA (RNA, protein-DNA complex) solution (0.1µg/ml) in the middle of an AP-mica substrate (usually $1x1cm^2$, or the size of the sample puck) for 5 minutes. Then rinse the surface thoroughly to remove all buffer components and dry (i.e., blow with dry gas or dry in vacuum). The sample is ready for imaging.

2. **The Immersion Procedure.** Immerse pieces of AP-mica into 0.1µg/ml DNA solutions (RNA, nucleoprotein complexes) and allow the sample to adsorb for 30-60 minutes. Take the strips out of the solution, rinse with water and dry (see above). The sample is ready for imaging.

   Note: This procedure is convenient if deposition needs to be done in strict temperature-controlled conditions. In this case, incubate the solution at a pre-set temperature for 10-15 minutes.

   Note: DNA concentration may be increased if molecules as small as several hundred base pairs (bp) are deposited. Otherwise, we recommend to decrease the DNA concentration if the length is more than 20kbp. For example, concentration of lambda DNA (~48kb) should be about 0.01µg/ml [1,4].

331.7.4 Imaging Conditions

The prepared samples can be imaged in air (dry atmosphere is recommended), in propanol or under water (cf. [1,3,4]). There is no special requirement for imaging procedures.

331.7.5 General Comments

A large range of concentrations can be used with AP-mica [1,2,5-9]. Deposition can also be performed in a wide range of temperatures (0 - 60°C, [2,5]) and pH. These are unique features in comparison with other sample preparation methods for AFM [5-9] and are very important for sample depositions, which are stable at specific environmental conditions. AP-mica is very stable; this allows, for example, preparation of the substrate well in advance of deposition. Also, once deposited onto AP-mica the samples are stable for several months if they are stored at ambient conditions.

331.7.6 References

1. Lyubchenko, Y.L., Gall, A.A., Shlyakhtenko, L.S., Harrington, R.E., Oden, P.I., Jacobs, B.L. and Lindsay, S.M. (1992), "Atomic force microscopy imaging of double stranded DNA and RNA," J. Biomolec. Struct. Dyn., 9, 589-606.

2. Lyubchenko, Y.L., Jacobs, B.L. and Lindsay, S.M.(1992), "Atomic force microscopy of reovirus dsRNA: a routine technique for length measurements," Nucl. Acids Res., 20, 3983-3986.

3. Lyubchenko, Y.L., Shlyakhtenko, L.S., Harrington, R.E., Oden, P.I. and Lindsay, S.M. (1993), "Atomic force microscopic demonstration of DNA looping by GalR and HU," Proc. Natl. Acad. Sci. USA, 90, 2137-2140.

4. Lyubchenko, Y.L., Oden, P.I., Lampner, D., Lindsay, S.M., and Dunker, K. (1993), "Atomic force microscopy of long DNA and bacteriophage in air, water and propanol: the role of adhesion forces," Nucleic Acids Res., 21, 1117-1123.

5. Lyubchenko, Y.L., Jacobs, B.L., Lindsay, S.M., Stasiak, A. (1995), "Atomic Force Microscopy of Protein-DNA Complexes. (Review article)," Scanning Microscopy, 9, 705-727.

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8. Lyubchenko, Y. L. (1997), "Functionalized AP-mica: Immobilization of DNA, RNA and Nucleoprotein Complexes for Imaging with AFM," In: Proceeding of the Fourth International Workshop: STM-AFM-SNOM: New Nanotools for Molecular Biology. Foundation Fourmentin-Guilbert. Noisy Le Grand, France. pp. VII-3 - 5.

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331.7.7 Comments Regarding Yuri Lyubchenko's Recipe

It is very important to use fresh APTES. With older bottles, samples fail to stick to it and the surface is unusually rough. When not in use, store the APTES bottle in a desiccator in the refrigerator. Flood the bottle with dry argon before replacing the cap. Also, be sure that you use dry argon when preparing the AP-mica and also when storing it. Use chromatographic grade argon and pass it through a molecular sieve drying column. Use a real vacuum pump. Lab vacuum ports are typically too weak (~15 inches Hg rather than the needed ~28 inches Hg). A small 4 inch glass desiccator (Sigma #D1920) can be used instead of a 2l desiccator. Premount the mica on a metal sample puck. Immediately before processing, cleave the mica and place it on a magnet mounted to the top of the desiccator.

It is also possible to lay the mica face down right on top of an eppendorf cap containing the APTES rather than mounting it up toward the top of the desiccator. In this case the processing time can be reduced to about 15 minutes. Using the N,N-diisopropylethylamine is also optional. According to Yuri Lyubchenko it renders the atmosphere as neutral as possible since the APTES is hydrolyzed at acidic pH, but it seems not to be essential. You may also try processing the mica for longer than the suggested 1-2 hours. This may make it more rough, however. Optimize the time for your own system since such factors as desiccator size and vacuum strength affect the product.

331.8 Sources of Mica

Spruce Pine Mica
Telephone: 828-765-4241
Fax 828-765-7192

Ted Pella, Inc.
P.O. Box 492477, Redding, CA 96049-2477
Telephone: 530-243-2200; 800-237-3526

We recommend that you do not cut mica with scissors but punch it out into discs using a punch set (e.g., available through McMaster-Carr). This prevents the deterioration of the crystal. We use 5 minute epoxy in our lab to glue punched mica discs to steel sample pucks.

331.9 Poly-L-Lysine Coating

To prepare poly-L-lysine adhesive for binding cells to a petri dish:

1. Use a 0.01% Poly-L-Lysine solution (e.g., Sigma #P-4832) in water. Polylysine has a molecular weight range of 150k-300k and should be stored in a refrigerator.

2. Incubate 50µL of the solution, or enough to cover the desired area, on a clean 12mm round coverglass.

3. Incubate for 15 minutes and then rinse with water or PBS.

   Note: It is also possible to use lower weight poly-l-lysine (~30k) and incubate for 30 minutes.