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Protocol

Micropatterning Cell-Substrate Adhesions Using Linear Polyacrylamide as the Blocking Agent

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Adapted from *Live Cell Imaging*, 2nd edition (ed. Goldman et al.). CSHL Press, Cold Spring Harbor, NY, USA, 2010.

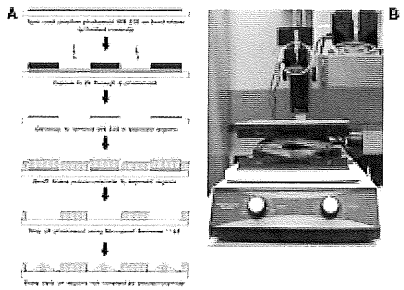
INTRODUCTION

Recent developments in micropatterning techniques have facilitated systematic investigations of how cells respond to geometric cues, which likely have an important role in many physiological processes, such as growth and differentiation. Micropatterned substrates also offer technical advantages for cell biological studies by normalizing cell shapes and reducing the variability among cultured cells. In this article, we present a simple, economical micropatterning method that may be easily implemented in most laboratories. We also describe how to treat the substrate with linear polyacrylamide (PAA) to inhibit unwanted cell adhesion. First, glass coverslip surfaces are activated with 3-methacryloxypropyltrimethoxysilane (bind-silane), an approach that was originally used for covalently linking PAA gels to glass plates for SDS-PAGE to prevent gels from shrinking and swelling during staining/destaining. The activated surfaces are then coated with the photoresist SPR-220, exposed to ultraviolet (UV) light through a photomask, and developed to selectively remove UV-exposed photoresist. Finally, linear PAA is grafted onto the exposed areas of the bind-silane substrate (to prevent cell adhesion), and the remaining photoresist is removed to uncover the glass surface, making the unexposed areas available as adhesive areas for cell culture.

RELATED INFORMATION

An outline for micropatterning cells using linear PAA and the removable positive photoresist SPR-220 is depicted in Figure 1A. The flowchart shows the sequence of steps for masking coverslips with photoresist, followed by the grafting of linear PAA onto the regions left exposed. This approach takes advantage of the simple grafting chemistry of linear PAA (Garoff and Ansorge 1981) and its ability to inhibit cell adhesion as well as the high resolving power and ease of removal of positive photoresists. The equipment setup required for photolithography is shown in Figure 1B.

Figure 1. Principle (*A*) and equipment (*B*) for micropatterning by photoresist stripping and linear PAA grafting. (*Inset*) High-flux 365-nm UV LED. An arrow points to the heat sink, and the oval represents the high-flux LED-based illuminator. The assembly of coverslip and photomask is placed on an adjustable stand, which is in turn placed on a shaker.



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MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

Reagents

Acrylamide solution (40% w/v)

Store at 4°C.

Ammonium peroxydisulfate (APS)

Store APS desiccated at room temperature. Prepare fresh 10% APS solution just before use.

Bind-silane working solution

Cells of interest

Developer (Microposit MF-319; MicroChem Corp.)

An alternative homemade developer is 2.45% tetramethylammonium hydroxide solution (TMAH) combined with 0.1% Triton X-100 (TMAH working solution).

ECM coating solution (optional; see Step 19)

Ethanol (95%; ACS/USP grade)

HEPES (1 M, pH 8.5)

Filter and store at 4°C.

Microspheres, carboxylate-modified (0.1 µm FluoSpheres; Molecular Probes/Invitrogen) (for estimating thickness of PAA layer; see Step 15)

N,*N*,*N*',*N*'-tetramethylethylenediamine (TEMED)

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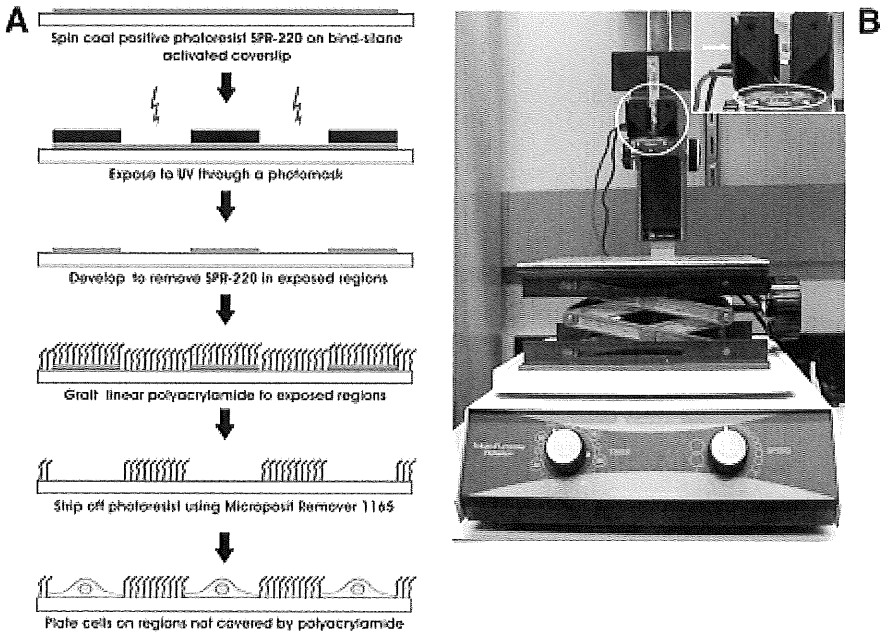

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Phosphate-buffered saline (PBS, pH 7.4)

Photoresist, positive (SPR-220.3; MicroChem Corp.)

Remover (Microposit 1165; MicroChem Corp.)

Equipment

Bunsen burner

Clamps

Coverslips

Typically, 45 x 50-mm no. 1 coverslips are used as the substrate. Smaller coverslips, matching the area of the pattern (e.g., 25 x 25-mm), are used as the top to control the spread area of linear PAA.

Diamond-tip pen

Heat block preset to 115°C

High-flux UV light-emitting diode (LED) (Opto Technology, Inc.)

Micropipettor and tips

Microscope with calibrated focusing mechanism (for estimating thickness of PAA layer; see Step 15)

Observation chamber to be used for cell culture

Orbital shaker

Petri dish, glass

Photomask with the desired pattern

If the experiment requires a pattern resolution better than 10 μm, then custom order the photomask (e.g., from Advance Reproductions Corp.). Otherwise, the mask may be ordered as an inexpensive transparency film from a number of companies (e.g., CAD/Art Services).

Razor blade or fine forceps

Spin processor (Laurell)

Alternatively, use a low-speed benchtop centrifuge modified to hold coverslips and placed in a chemical fume hood for spin coating.

Tissues (laboratory)

METHOD

Activation of Coverslips (20 min)

1. Mark one of the corners of each 45x50-mm no. 1 coverslip with a diamond-tip pen to facilitate later identification of the activated side.

2. Pass each coverslip through the inner flame of a Bunsen burner (with the marked side facing down) to increase its hydrophilicity.

3. Apply the bind-silane working solution as follows:

i. Working in a fume hood, apply ~30 µL of bind-silane working solution onto the flamed side of each coverslip and spread it evenly over the coverslip using the pipette tip.

ii. Remove any excess with tissues.

iii. Allow bind-silane to react for 3 min.

4. Rinse the coverslips with ethanol and wipe them with a tissue to remove any residual reagent. Allow the coverslips to air-dry.
Activated coverslips are stable for at least 3 mo at room temperature.

Photolithography (20 min)

5. Bake an activated coverslip on a heat block for 2 min at 115°C. Cool the coverslip to room temperature.

6. Working in a fume hood, deposit 180 µL of positive photoresist SPR-220 onto each 45x50-mm coverslip. Spread the photoresist uniformly across the activated side of the coverslip in a spin processor at 5000 rpm for 30 sec.
A benchtop centrifuge modified to hold coverslips will generate similar results.

7. Bake the coverslip for 90 sec at 115°C.
After baking, photoresist-coated coverslips can be stored in the dark for up to 3 mo at room temperature.

8. Prepare the photomask/coverslip assembly:

i. Using the marks made with the diamond pen (in Step 1) as a guide, orient the coverslip so that the activated surface is facing up.

ii. Place the photomask over the coverslip.

iii. Sandwich the photomask and coverslip between two glass plates.

iv. Secure the assembly with clamps to ensure tight contact between the mask and the prepared surface of the coverslip.
Proper contact between the photomask and the substrate is particularly important if the light source is not well-collimated.

9. Expose the coverslip to 365-nm UV light from a high-flux UV LED at a distance of 3 cm for 45 sec while rotating the substrate.

If the light source is not uniform, place the photomask/coverslip sandwich onto an orbital shaker rotating at ~70 rpm during the UV exposure to create a uniform average illumination across the surface. The exact exposure time depends on the optical conditions and must be calibrated for each setup.

10. Bake the coverslip for 90 sec at 115°C. Cool the coverslip to room temperature.

11. Immerse the coverslip in Microposit developer MF-319 in a glass Petri dish inside a chemical fume hood, and shake it for 45 sec.
Optimal timing and mixing conditions are affected by the exposure conditions and should be carefully controlled. Developing in TMAH working solution generates comparable results.

12. Rinse the coverslip extensively in H_2O and allow it to air-dry.
Start Step 13 when processing for photolithography as described above, so that degassing of the polyacrylamide solution will be complete after finishing Step 12.

Blocking Nonadhesive Regions with Linear PAA (25 min)

We have found that short linear PAA minimally interferes with cell attachment to adhesive regions and yields excellent resolution, whereas long linear PAA maximally blocks cell attachment to nonadhesive regions. However, long PAA chains appear to cause a slight delay in cell attachment, possibly by dangling across and partially covering the surface of adhesive regions. The length of the PAA chains can be controlled by changing the concentration of the initiators, TEMED and APS, during polymerization. For example, polymers formed with 1% TEMED and 8% acrylamide are able to block cell adhesion for >2 wk, whereas polymers formed with 3% TEMED and 8% acrylamide last for ~2 d, which is still sufficient for many experiments. Eventually, cells are able to break out of the confined area and spread over the entire glass surface.

13. Prepare a mixture of 200 µL of 40% acrylamide, 10 µL of 1 M HEPES (pH 8.5), and 752.5 µL of H_2O to obtain a final concentration of 8% acrylamide. Degas the solution for 20 min.

14. Prepare the PAA layer:

i. After degassing, add 15 µL of TEMED and 22.5 µL of 10% APS to the acrylamide solution, mix thoroughly, and pipette it immediately onto the glass surface patterned with SPR-220.

ii. Place a coverslip over the acrylamide (it should be large enough to cover the micropattern; we typically use a 25 x 25-mm coverslip).

iii. Allow the acrylamide to polymerize for 20 min.

15. Carefully remove the top coverslip using either a razor blade or fine forceps. Rinse the surface thoroughly with H_2O to remove any unreacted acrylamide.
Linear PAA-coated coverslips may be stored in H_2O at least overnight.
To estimate the thickness of the linear PAA layer, 10 µL of 0.1-µm fluorescent microspheres may be added to the acrylamide solution. The beads are trapped by PAA and serve as markers during optical sectioning. The thickness of the PAA layer can be estimated from the distance between the highest and lowest plane where beads are found in focus, using a calibrated microscope focusing mechanism.

Removal of SPR-220 to Expose Adhesive Regions (15 min)

16. Remove the SPR-220, which has been patterned by UV exposure and development, as follows:

i. Immerse the substrate coverslip in H_2O for 1 min with agitation.

ii. Incubate the coverslip in Microposit remover 1165 for 5 min at room temperature or until the coverslip becomes clear.

17. Wash the coverslip surface thoroughly with H_2O to remove Microposit remover. Allow the surface to air-dry.
Surface micropatterning may be observed as preferential wetting of the area covered with PAA when rinsing gently with H_2O.
At this stage, the micropatterned coverslip is stable and may be stored for at least 3 mo at room temperature.
See Troubleshooting.

Coating Patterned Substrates with Extracellular Matrix Proteins (optional; ~45 min)

18. Mount the micropatterned coverslip in the observation chamber to be used for cell culture.

19. (Optional) Add extracellular matrix (ECM) coating solution, with fluorescent dye if necessary, and incubate with gentle shaking for at least 30 min at room temperature.
The fluorescent dye renders the micropattern visible under fluorescence optics. Without the dye, the adhesive pattern may be detectable only through cell behavior.

20. Rinse the substrate three times with PBS.

21. Sterilize the patterned surface of the coverslip under UV light for 15 min.

22. Plate the cells onto the micropatterned coverslip.
See Troubleshooting.

TROUBLESHOOTING

Problem: Patterning is poor.

[Step 17]

Solution: Consider the following:

1. Make sure that the side coated with SPR-220 is facing the patterned side of the photomask during exposure.

2. Make sure that the contact between the photomask and coverslip in Step 8 is tight.

3. After photolithography, examine the substrate under a microscope and adjust the exposure and development times accordingly. Over- or underdevelopment causes the photoresist-covered region to be larger or smaller than the pattern on the photomask, respectively, whereas poor contact between the

photomask and the coverslip causes fuzzy edges. Adjust the time of development by trial and error so that it is between 30 and 60 sec.

Problem: PAA-bound SPR-220 comes off as a sheet instead of gradually dissolving in Microposit remover 1165 solution.

[Step 17]

Solution: This problem is almost exclusively due to underdevelopment of the photoresist. Consider the following:

1. After exposure and development, examine the pattern under a microscope to make sure that the exposed region is clear. Residual SPR-220 makes the coverslip appear cloudy and causes linear PAA to coat onto a thin film of SPR-220 instead of the activated coverglass surface. Extend the development time and/or exposure time accordingly.

2. Check the expiration date of Microposit developer MF-319 and order a new batch if necessary.

3. TMAH working solution may gradually become less effective after 1 mo. Prepare fresh TMAH working solution if necessary.

Problem: Cells do not appear to be conforming to the micropattern.

[Step 22]

Solution: If linear PAA coats residual SPR-220 rather than the glass surface of the coverslip, then in Step 17 it will come off as a sheet. After photolithography, carefully examine the coverslip under a microscope to confirm that the region free of SPR-220 is clear. Any residual SPR-220 on the coverslip appears cloudy. This problem may be resolved by extending development or exposure time.

Problem: Cell attachment is poor.

[Step 22]

Solution: The amounts of TEMED and APS required are different from those typically used in preparing PAA gels. Insufficient TEMED and APS generate long linear polymers of PAA that might extend onto adhesive areas, thus blocking or interfering with cell attachment to the adhesive areas. Adjust the amounts of TEMED and APS used to polymerize the acrylamide.

DISCUSSION

Most biologists have avoided using micropatterned substrates, because, as described in published procedures (LeDuc et al. 2002), the costs are high and the necessity of using a clean room and special equipment for photolithography, metal coating, reactive ion etching, and plasma treatment is impractical. Tsai et al. (2007) have shown that a clean room is not necessary for most biological applications because the experiments tolerate minor defects in patterning. We have developed methods to eliminate the need for any special and expensive equipment. One approach, reported previously, used the negative photoresist SU-8, micropatterned by photolithography, as a substrate for cell culture (Wang et al. 2006; Mader et al. 2007; Guo and Wang 2007).

However, SU-8 shows strong autofluorescence when excited at 488 nm. Although positive photoresists such as SPR-220 also suffer from strong autofluorescence when excited near 546 nm, the different chemical principle involved permits them to be removed much more easily with solvents.

The micropatterning strategy described here leverages on the ability to easily strip off positive photoresists such as SPR-220 (Fig. 1A). The most costly piece of equipment required is a spin coater for coating the photoresist, which costs <$5000 and may be replaced with a modified benchtop centrifuge. Expensive photomask aligners are avoided by using contact exposure of photoresists, and UV lamps with collimation optics are replaced with an inexpensive and long-life UV LED (<$200) and an orbital shaker to achieve uniform illumination. This approach is capable of generating nearly perfect patterns with a resolution of ~1 μm (Figs. 2A, 3B), limited primarily by the resolution of the photomask. Actin, microtubules, and focal adhesions showed the characteristic organization on square or rectangular islands (Fig. 2B–E). In addition, there is no detectable effect of bind-silane treatment on the adhesion or behavior of cells on the glass surface. In addition to the present method, linear PAA may also be used in conjunction with other approaches of micropatterning, such as microcontact printing, as a robust blocking reagent. For example, adhesive proteins may be applied to a glutaraldehyde-activated glass surface (Wang and Pelham 1998) by microcontact printing. The rest of the activated surface can then be blocked by grafting PAA (Fig. 3A).

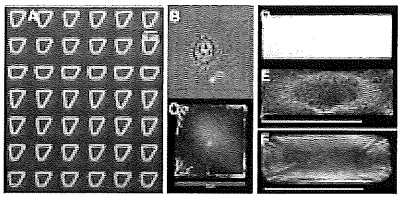

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Figure 2. Micropatterns generated by photoresist stripping and linear PAA grafting. The pattern generated with SPR-220 photoresist is visible in phase-contrast optics (*A*), but it becomes invisible upon stripping the photoresist (*B*). However, it remains easily detectable with Alexa Fluor-conjugated fibronectin (*D*). NIH-3T3 cells plated on a square pattern show a confined shape in phase-contrast images (*B*). Focal adhesions (*C*) and actin bundles (*F*), labeled with antivinculin antibodies and fluorescent phalloidin, respectively, show the characteristic concentration at the corners of the square or rectangle. Staining with antitubulin antibodies shows a dense network (*E*). Bar, 50 μm.

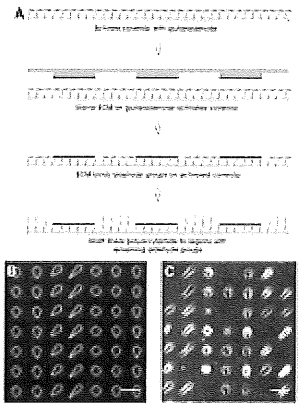

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Figure 3. Patterning by microcontact printing and linear PAA grafting. The method involves microcontact printing of matrix proteins on an activated glass surface, followed by grafting of linear PAA as the blocking reagent for the remaining areas (*A*). The pattern as seen on the polydimethylsiloxane (PDMS) stamp (*B*) is transferred to the culture surface to confine the cell shape (*C*). This method generates more defects than the photoresist stripping method because of the inconsistencies in microcontact printing. Bar, 40 μm.

Compared with other blocking agents (Nelson et al. 2003; Falconnet et al. 2004), long-chain PAA blocked cell adhesion for substantially longer periods. For example, neither polyethylene glycol derivatives nor pluronic F-108 shows reliable resistance to cell adhesion (W.-h. Guo and Y.-l. Wang, unpubl.). Only surfactant pluronic F-

127 has been reported to have a blocking stability similar to long-chain PAA (Tan et al. 2004). Although small air bubbles worked effectively as an adhesion blocker (Wang et al. 2006), the application was limited by the compromise of optic quality caused by their strong reflection. Additional advantages of the present method include a high resolution of 1–5 μm, minimal autofluorescence, and no need for gold coating, which causes quenching of fluorescence, particularly in total internal reflection fluorescence microscopy (Kandere-Grzybowska et al. 2005; Falconnet et al. 2006). Thus, this simple, economical strategy will allow micropatterning to be used in a wide array of cell biological studies, including high-throughput screening of conditions that affect cell-shape control.

ACKNOWLEDGMENTS

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Recipe

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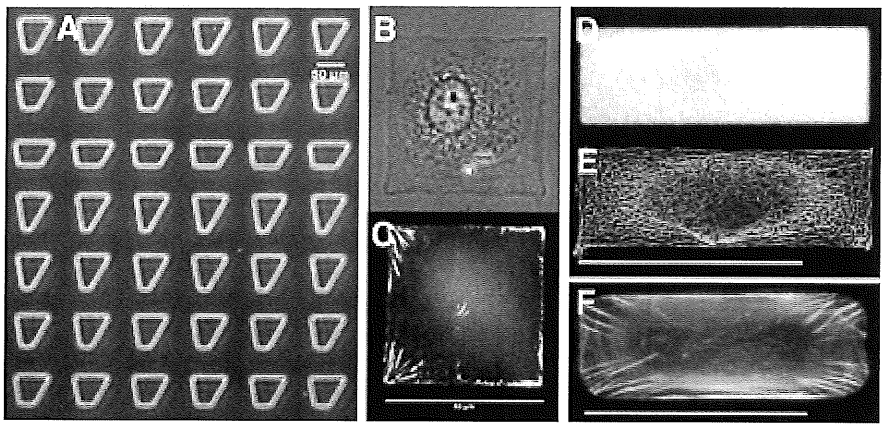

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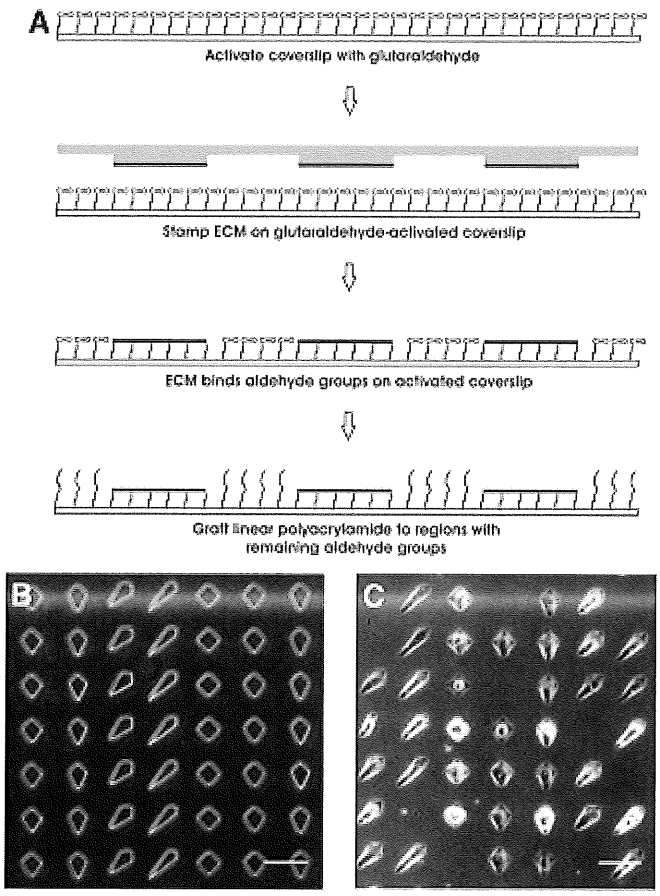

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Bind-silane working solution

Reagent	Quantity
Bind-silane	3 µL
Ethanol (95%)	950 µL
Acetic acid (glacial)	50 µL

Bind-silane is available from GE Healthcare (17-1330-01) or Sigma-Aldrich (M6514).

Recipe

ECM coating solution

Reagent	Quantity (for 1 mL)
Fibronectin (1 mg/mL)	10 µL
HEPES (1 M; pH 8.5)	10 µL
H_2O	980 µL

To make the solution fluorescent, add 1 µL of either Alexa Fluor 488 or 568 dye (Molecular Probes/Invitrogen). Prepare in dimethyl sulfoxide (DMSO) at 10 mg/mL and store at –20°C; avoid exposure to light.

Fibronectin can be replaced with any extracellular matrix (ECM) protein of interest (e.g., collagen or laminin).

Recipe

Phosphate-buffered saline (PBS)

Reagent	Amount to add (for 1X solution)	Final concentration (1X)	Amount to add (for 10X stock)	Final concentration (10X)

NaCl	8 g	137 mM	80 g	1.37 M
KCl	0.2 g	2.7 mM	2 g	27 mM
Na_2HPO_4	1.44 g	10 mM	14.4 g	100 mM
KH_2PO_4	0.24 g	1.8 mM	2.4 g	18 mM

If necessary, PBS may be supplemented with the following:

$CaCl_2 \cdot 2H_2O$	0.133 g	1 mM	1.33 g	10 mM
$MgCl_2 \cdot 6H_2O$	0.10 g	0.5 mM	1.0 g	5 mM

PBS can be made as a 1X solution or as a 10X stock. To prepare 1 L of either 1X or 10X PBS, dissolve the reagents listed above in 800 mL of H_2O. Adjust the pH to 7.4 (or 7.2, if required) with HCl, and then add H_2O to 1 L. Dispense the solution into aliquots and sterilize them by autoclaving for 20 min at 15 psi (1.05 kg/cm^2) on liquid cycle or by filter sterilization. Store PBS at room temperature.