

Formaldehyde Fixation of Extracellular Matrix Protein Layers for Enhanced Primary Cell Growth

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[Abstract] Coating tissue culture vessels with the components of the extracellular matrix such as fibronectin and collagens provides a more natural environment for primary cells *in vitro* and stimulates their proliferation. However, the effects of such protein layers are usually rather modest, which might be explained by the loss immobilized proteins due to their weak non-covalent association with the tissue culture plastic. Here we describe a simple protocol for a controlled fixation of fibronectin, vitronectin and collagen IV layers by formaldehyde, which substantially enhances the stimulation of primary cell proliferation by these extracellular proteins.

Keywords: Mesenchymal stem cells, Fibronectin, Vitronectin, Collagen IV, Formaldehyde, Fixation

[Background] The components of the extracellular matrix (ECM) such as fibronectin, laminin, vitronectin and collagens are often used for coating tissue culture vessels since they provide a more natural environment for primary cells *in vitro* and stimulate their proliferation (Sawada *et al.*, 1987; Rajaraman *et al.*, 2013). However, the observed stimulation of cell proliferation by these protein layers is usually fairly modest. This might be explained by their weak non-covalent association with the tissue culture plastic resulting in delamination and loss of immobilized protein molecules. Recently, it has been shown that the retention of ECM produced by the cells might be significantly increased by covalent immobilization of fibronectin to the plastic surface (Prewitz *et al.*, 2013). However, ECM production is cumbersome and may be difficult to standardize. We have demonstrated that simple formaldehyde fixation under controlled conditions of layers formed by the selected individual ECM proteins can substantially enhance positive effects of these proteins on cell proliferation (Andreeva *et al.*, 2016). Here we describe a detailed protocol of culture plastic coating and formaldehyde fixation for three individual components of ECM, namely fibronectin, vitronectin and collagen IV. Although we did not test the effects of controlled fixation with other protein constituents of the ECM, the positive results obtained with our protocol for three proteins of vastly differing molecular and biological properties provide sufficient reasons to assume the general applicability of the described procedure for enhancing proliferation stimulatory properties of a wider range of ECM proteins.

Materials and Reagents

1. 3.5 cm cell culture dish (Greiner Bio One International, CELLSTAR[®], catalog number: 627160)
2. Sterile pipette filter tips 200 and 1,000 μ l (Greiner Bio One International, catalog numbers: 739288 and 740288, respectively)
3. Parafilm M (Sigma-Aldrich, catalog number: P7668)
4. 15 ml centrifuge tube (Greiner Bio One International, CELLSTAR[®], catalog number: 188261)
5. 50 ml centrifuge tube (Greiner Bio One International, CELLSTAR[®], catalog number: 227261)
6. 1.8 ml round bottom cryogenic tubes (Thermo Fisher Scientific, Thermo Scientific[™], catalog number: 375418)
7. 0.22 μ m syringe filter (GVS, catalog number: 1213641)
8. 10 and 20 ml syringes (SFM Hospital Products, catalog numbers: 534235 and 534236, respectively)
9. 2 and 10 ml Serological pipets (Greiner Bio One International, CELLSTAR[®], catalog numbers: 710180 and 607107, respectively)
10. Phosphate-buffered saline (PBS), pH 7.4 tablets (Thermo Fisher Scientific, Gibco[™], catalog number: 18912014)
11. Paraformaldehyde (Sigma-Aldrich, catalog number: 76240)
Note: This product has been discontinued.
12. Sodium hydroxide (NaOH), 10 M (Sigma-Aldrich, catalog number: 72068)
13. Fibronectin human (Imtek, catalog number: H Fne-C)
14. Vitronectin human (Imtek, catalog number: H Vne-C)
15. Collagen IV bovine (Imtek, catalog number: B C44-C)
16. Acetic acid (Sigma-Aldrich, catalog number: 695092)
17. Ethanol 96% (Sigma-Aldrich, catalog number: 24105)
Note: This product has been discontinued.
18. Sterile distilled water
19. 1x phosphate-buffered saline (PBS) pH 7.4 (see Recipes)
20. Formaldehyde solution (see Recipes)
21. Human fibronectin solution (prepare freshly) (see Recipes)
22. Human vitronectin solution (prepare freshly) (see Recipes)
23. Bovine collagen IV solution (prepare freshly) (see Recipes)

Equipment

1. Pipette controller (Corning, catalog number: 4091)
2. Automatic single-channel pipettes, 20-200 and 100-1,000 μ l (Gilson-compatible)
3. Magnetic hot plate stirrer (Sigma-Aldrich, catalog number: Z168580)
4. Magnetic stirring bar, 7 x 2 mm (Fisher Scientific, Fisherbrand[™], catalog number: 14-513-63)

5. Laminar flow tissue culture hood
Note: This item can be ordered from any qualified company.
6. Refrigerator with interior power outlets
Note: This item can be ordered from any qualified company.
7. CO₂ incubator (Sanyo, catalog number: MCO-18AIC)
Note: This product has been discontinued. Possible substitute: Panasonic Healthcare, model: MCO-18AC.
8. Centrifuge 5810 R (Eppendorf, model: 5810 R, catalog number: 5811000320)
9. Autoclave
Note: This item can be ordered from any qualified company.

Procedure

A. Coating Petri dishes with fibronectin followed by formaldehyde fixation

Note: All procedures below should be performed under a laminar flow hood.

1. Add 1 ml of the 50 µg/ml fibronectin solution (see Recipes) to each of the 3.5-cm Petri dishes. Make sure the entire dish surface is completely covered by gently swirling the solution or redistributing it over the surface using pipette tip.
Note: The fibronectin layer is only about 1-mm deep for the indicated volume, thus it is recommended to perform this operation with Petri dishes placed on a strictly horizontal surface to ensure even coverage of all areas of the dish.
2. Incubate dishes with the lid on for 1.5 h at 37 °C in a humidified CO₂ incubator.
3. Aspirate carefully and save the fibronectin solution.
Note: The saved fibronectin solution can be re-used at least once. Store it at 4 °C and use within one week.
4. Add 1 ml of 0.4% formaldehyde solution (see Recipes). Incubate dishes with the lid on for 20 min under a sterile laminar flow hood.
5. Carefully aspirate the formaldehyde solution, wash twice with 2 ml of sterile PBS and then 4 times with 4 ml of sterile PBS.
Note: Formaldehyde solution should be disposed of as a hazardous waste.
6. Dry dishes for 1 h under the laminar flow hood.
Note: Fibronectin-covered dishes can be sealed with Parafilm and stored at 4 °C for up to 1 week before use.

B. Coating Petri dishes with vitronectin followed by formaldehyde fixation

Note: All procedures below should be performed under a laminar flow hood.

1. Add 0.5 ml of the 10 µg/ml vitronectin solution (see Recipes) to each of the 3.5-cm Petri dishes. Make sure the entire dish surface is completely covered by gently swirling the solution or redistributing it over the surface using pipette tip.

Note: The vitronectin layer is only about 0.5-mm deep for the indicated volume, thus it is recommended to perform this operation with Petri dishes placed on a strictly horizontal surface to ensure an even coverage of all areas of the dish.

2. Incubate dishes with the lid on for 1.5 h at 37 °C in a humidified CO₂ incubator.
3. Aspirate carefully and save the vitronectin solution.

Note: The saved vitronectin solution can be re-used at least once. Store it at 4 °C and use within one week.

4. Add 1 ml of 0.4% formaldehyde solution (see Recipes). Incubate dishes with the lid on for 20 min under a sterile laminar flow hood.
5. Carefully aspirate the formaldehyde solution, wash twice with 2 ml of sterile PBS and then 4 times with 4 ml of sterile PBS.

Note: Formaldehyde solution should be disposed of as a hazardous waste.

6. Dry dishes for 1 h under the laminar flow hood.

Note: Vitronectin-covered dishes can be sealed with Parafilm and stored at 4 °C for up to 1 week before use.

C. Coating Petri dishes with collagen IV followed by formaldehyde fixation

Note: Coating Petri dishes overnight is performed in the refrigerator, the rest of procedures should be performed under a laminar flow hood while keeping collagen solution on ice and minimizing exposure to room temperature conditions.

1. Add 0.5 ml of the 50 µg/ml collagen IV solution (see Recipes) to each of 3.5-cm Petri dishes. Make sure the entire dish surface is completely covered by gently swirling the solution or redistributing it over the surface using pipette tip. Immediately transfer the dishes to the refrigerator.
2. Incubate dishes with the lid on overnight at 4 °C.

Note: The collagen layer is only about 0.5-mm deep for the indicated volume, thus it is important to perform this operation with Petri dishes placed on a strictly horizontal surface to ensure even coverage of all areas of the dish.

3. Transfer dishes to the laminar flow hood, carefully aspirate and save collagen solution.

Note: The saved collagen solution can be re-used at least once. Store it at 4 °C and use within one week.

4. Add 1 ml of 0.4% formaldehyde solution (see Recipes). Incubate for 20 min under sterile laminar flow hood.
5. Carefully aspirate the formaldehyde solution, wash twice with 2 ml of sterile PBS and then 4 times with 4 ml of sterile PBS.

Note: Formaldehyde solution should be disposed of as a hazardous waste.

6. Dry dishes for 1 h under the laminar flow hood.

Note: Fibronectin-covered dishes can be sealed with Parafilm and stored at 4 °C for up to 1 week before use.

Data analysis

The assessment of the success of ECM protein coating and fixation includes analysis of MSC or other primary cell proliferation rate. Proliferation rates are determined by seeding 15 to 30 thousand cells per 3.5-cm dish followed by culturing for 1 or 2 days. A minimal testing setup includes: a) control untreated dishes; b) dishes, coated with ECM protein but unfixed; c) dishes coated with ECM and fixed with formaldehyde. Each variant is performed in triplicate, cells after culturing are collected by trypsinization followed by manual or automatic cell counting. It is recommended to perform counting of each cell sample two or three times using independent cell aliquots to increase the accuracy of analysis. It is important to assess the cell proliferation while the cells are within the exponential growth phase, that is, with cell confluence not exceeding 50%. The proliferation rate can be represented as an expansion factor (ratio of harvested to seeded cells) or population doublings (\log_2 of expansion factor). The results are analyzed by determining mean, standard deviations and performing Student's *t*-tests. The typical result of such analysis is shown in Figure 1.

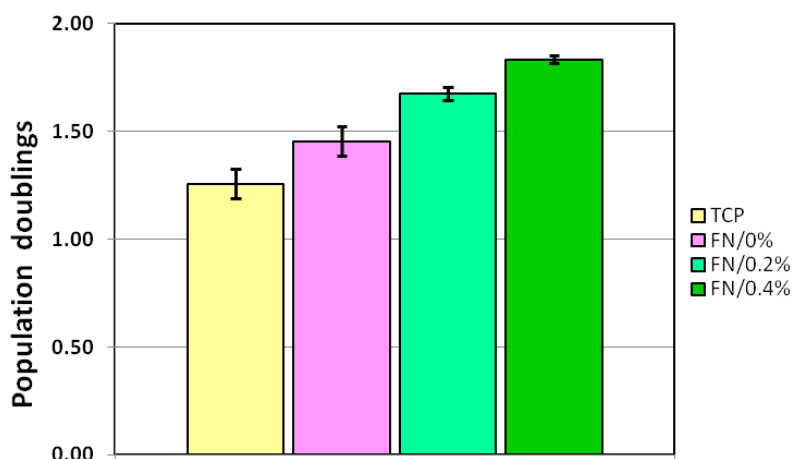


Figure 1. Controlled fibronectin fixation significantly enhances expansion of mesenchymal stem cells (MSCs). Mouse MSC growth under hypoxia conditions (5% O₂) on control uncoated dishes (TCP) and dishes coated with fibronectin at a concentration of 50 µg/ml. Fibronectin-coated dishes were left unfixed (FN/0%) or fixed with 0.2% formaldehyde (FN/0.2%) or 0.4% formaldehyde (FN/0.4%). Y axis depicts the number of population doublings underwent by the culture within 24 h. Data are presented as mean ± SD.

Notes

1. Imtek is a Russian company producing, among others, extracellular matrix proteins for cell culture. Their website: <http://new.imtek.ru/eng>. We have as yet no experience with the extracellular matrix proteins from other companies.

2. The formaldehyde concentrations producing best results in our setup for 20-min fixation time are 0.3%-0.4%. Higher formaldehyde concentrations result in diminished effects on cell proliferation (Andreeva *et al.*, 2016). When working with other extracellular matrix proteins not tested by us, it is highly advisable to perform test fixations with formaldehyde concentrations ranging from 0.2% to 0.6% to find the optimal fixation conditions.
3. The measured temperature during formaldehyde fixation (*i.e.*, under laminar flow hood) in our setup is 24 °C to 26 °C. Since the level of fixation depends on the temperature, it is recommended to determine the temperature under the laminar hood to make sure the deviation from the above temperature does not exceed 2 °C. If the temperature is out of this range, it is recommended to perform test fixations for somewhat different periods of time to determine the optimal fixation time for a given experimental setup.
4. According to our results (Andreeva *et al.*, 2016), coating Petri dishes with fibronectin at the 50 µg/ml concentration produced better results as compared to the 10 µg/ml concentration, whereas increasing fibronectin concentration to 250 µg/ml produced only a small additional effect. Although we did not test this yet, it is fairly possible that extending time for coating dishes over the 1.5-h period used in our protocol may produce a sizeable increase in the effects of fibronectin layers at 10 µg/ml concentration.
5. Coating with vitronectin, due to a high cost of this protein, was performed at lower concentration (10 µg/ml) as compared to fibronectin and collagen IV (50 µg/ml), and produced smaller effects than those observed for the latter two proteins (Andreeva *et al.*, 2016). Based on analogy with fibronectin results, it is likely that increasing concentration of vitronectin during plastic coating will produce larger effects.
6. Since the treatment of plastic surfaces for different types vessels and by various manufacturers may differ, it is fairly possible that the effects of fixed extracellular matrix protein layers on cell proliferation may vary. In our hands, fibronectin coating followed by fixation was more efficient for 3.5-cm Petri dishes than for 6-well plates.
7. Formaldehyde during storage may polymerize at lower temperatures and decompose at higher temperatures, thus decreasing the effective formaldehyde concentration. It is therefore recommended to use the prepared formaldehyde solution within one week and, preferably, prepare formaldehyde solution freshly for each fixation experiment.
8. It is important that collagen IV is processed at low temperature to prevent its slow denaturation which occurs even at room temperature.
9. Among the cell types tested by us, the most pronounced effects were observed for mesenchymal stem cells, both murine and human ones, smaller effects were obtained for fibroblasts, and no positive effects were observed for established cell lines (Andreeva *et al.*, 2016).

Recipes

1. 1x phosphate-buffered saline (PBS) pH 7.4
 - 2 tablets PBS (10 g)
 - Add distilled water to a final volume of 1 L
 - Sterilize by autoclaving
 - Store at room temperature
2. Formaldehyde solution
 - Add 4.0 g paraformaldehyde to a beaker with magnetic stirring bar
 - Add distilled water to a final volume 100 ml
 - Boil until complete dissolution on a hot plate magnetic stirrer, cool down
 - Add 10 N NaOH dropwise to pH 7.4
 - Store at 5 °C, use within 1 week
 - Dilute prior to the experiment to the desired concentration (usually, 0.4%) with sterile 1x PBS
3. Human fibronectin solution (prepare freshly)
 - 1 ml fibronectin sterile solution 1 mg/ml
 - 19 ml sterile 1x PBS
 - Store at 4 °C, use within one week
4. Human vitronectin solution (prepare freshly)
 - 0.25 mg sterile vitronectin dry powder
 - 25 ml sterile 1x PBS
 - Store at 4 °C, use within one week
5. Bovine collagen IV solution (prepare freshly)
 - Notes:*
 - i. *All manipulations should be performed at temperatures 0 °C to 5 °C using pre-chilled solutions.*
 - ii. *Sterilize the magnet before use in 70% ethanol for 1 h, then under UV irradiation for 1 h.*
 - a. Add 1.0 ml of 20 mM acetic acid (filter-sterilized) to 0.5 mg sterile collagen IV dry powder in a 1.8-ml round-bottom cryovial
 - b. Add a small-size magnetic stirring bar and stir the collagen solution overnight on a magnetic stirrer at 4 °C
 - c. Dilute the prepared collagen IV solution 10-fold with cold sterile 20 mM acetic acid to a final concentration of 50 µg/ml
 - d. Store the final solution at temperature 4 °C, use within one week

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