In vitro Fluorescent Matrix Degradation Assay for *Entamoeba histolytica*

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[Abstract] Fluorescent matrix degradation assay is a popular and widely used assay in the field of invadopodium biology (Artym *et al*., 2009). Matrix remodeling and degradation can be observed under both physiological and pathological conditions. Cancer cells extensively remodel and degrade the underlying matrix by employing actin-rich protrusive structures called invadosomes. Similar structures are formed by the protozoan parasite *Entamoeba histolytica* (*E. histolytica*), upon coming in contact with fibronectin, a major component of the host (extracellular matrix) ECM. Here, we describe a similar assay to measure matrix degradation by *Entamoeba histolytica.*

Materials and Reagents

1. Sterile disposable pipettes (10 ml and 5 ml)
2. Aluminum foil
3. Four well tissue culture plates (Thermo Fisher Scientific, Nunc™, catalog number: 176740)
4. Round Glass coverslips (12 mm) (Bellco Glass, catalog number: 1943-10012A)
5. Parafilm
6. *Entamoeba histolytica* HM1:IMSS strain (a kind gift from Prof. Alok Bhattacharya, School of Life Sciences, Jawaharlal Nehru University, New Delhi, India)
7. Fluorescently labeled fibronectin (CYTOSKELETON, catalog number: FNR02)
8. Glutaraldehyde (25% stock solution in dH_2O, EM grade) (Sigma-Aldrich, catalog number: G5882)
9. Sodium Borohydride ($NaBH_4$) (Sigma-Aldrich, catalog number: 452882)
10. Poly-L-lysine (Sigma-Aldrich, catalog number: P8920)
11. 70% ethanol (Merck Millipore Corporation)
12. Alexa 568 Phalloidin (Thermo Fisher Scientific, Molecular Probes™, catalog number: A12380)
13. 1% BSA (Sigma-Aldrich, catalog number: A-7906) in PBS
14. Mowiol mounting medium (Sigma-Aldrich, catalog number: 81381)
15. Complete BI medium (incomplete supplemented with 15%, Adult Bovine Serum)
16. GasPak™ EZ Anaerobe Pouch System (BD Biosciences, catalog number: 260683)
17. Sterile dH_2O
18. Sodium chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)

19. KCl
20. Potassium phosphate monobasic (Na_2HPO_4) (Sigma-Aldrich, catalog number: P5655)
21. Potassium phosphate dibasic trihydrate (KH_2PO_4) (Sigma-Aldrich, catalog number: P5504)
22. NaOH
23. Hilyte 488 Fibronectin
24. Biosate peptone BBL (BD Biosciences, catalog number: 211862)
25. Glucose (Dextrose) (Sigma-Aldrich, catalog number: D9434)
26. L-cysteine (Sigma-Aldrich, catalog number: C1276)
27. L-ascorbic acid (Sigma-Aldrich, catalog number: A5960)
28. Ferric ammonium citrate (Sigma-Aldrich, catalog number: F5879)
29. 10x phosphate-buffered saline (PBS) (see Recipes)
30. 0.01% poly-L-lysine solution (see Recipes)
31. 0.5% glutaraldehyde solution (see Recipes)
32. 5 mg/ml sodium borohydride (see Recipes)
33. 4% paraformaldehyde (PFA) (see Recipes)
34. 1% BSA in PBS (see Recipes)
35. 0.1% Triton-X-100 (see Recipes)
36. 100 µg/ml Hilyte488 Fibronectin (FNR02) (see Recipes)
37. Basal incomplete medium (see Recipes)
38. Complete medium (see Recipes)

Equipment

1. Laminar air flow hood
2. 37 °C incubator
3. Forceps
4. Moist chamber
5. Vacuum Pump and glass Pasteur pipettes with tubings
6. Table top centrifuge with swinging bucket rotor

Procedure

Video 1. *In vitro* fluorescent matrix degradation assay for *Entamoeba histolytica*

Coating of glass coverslips with fluorescent matrix

1. Clean the glass coverslips with acetone, air dry and sterilize them by autoclaving before use.
2. Further, handle the coverslips under sterile conditions inside the laminar air flow hood. Transfer the coverslips to a four well tissue culture plate using ethanol wiped forceps.
3. Coat each coverslip with 0.01% poly-L-lysine using 80 µl of poly-L-lysine solution per coverslip for 20 min at room temperature. Air dry the solution under the hood.
4. Wash the coverslips by adding 500 µl of sterile 1x PBS gently to the side of the well and incubating for 5 min each. The poly-L-lysine coated side should face up during the washes. After each wash aspirate it gently using a sterile glass pasteur pipette attached to a vacuum pump and repeat the washes for three times.
5. After the third PBS wash, add 500 µl of ice cold 0.5% glutaraldehyde to each well such that the coverslips are immersed and incubate under dark at room temperature for 15 min.
6. Aspirate the glutaraldehyde solution gently by tilting the plate to an angle such that coverslips are not disturbed and again wash with sterile 1x PBS. Repeat the washes for three times.
7. Make a humid chamber using tissue paper and 70% ethanol. Neatly make a stack of tissue paper by folding it over and moisten it by spraying with 70% ethanol. Place the stack in a sterile Petri dish to make a humid chamber. Place a neatly cut parafilm over the moist tissue paper stack and press it gently so that it sticks well.
8. Now, place appropriate number of drops of 50 µl of prewarm (37 °C, preferably in a water bath) fluorescently labeled fibronectin (100 µg/ml) over the parafilm according to the number of coverslips being coated.

9. Carefully lift the coverslips from the wells holding at the edges using a pair of forceps and place them with their poly-L-lysine coated side facing downwards on the drop of the fluorescent fibronectin on the parafilm. While placing the coverslips, ensure that there is no air bubble trapped beneath them. If so, gently press the coverslips with the forceps to eliminate the trapped air bubbles.
10. Incubate under safe light in a 37 °C incubator for an hour.
11. After an hour, remove the coverslips from the incubator. Now coverslips should be handled in safe light. Lift the coverslips carefully one-by-one with the help of the forceps without scratching the matrix and place them with their coated side facing up in a fresh tissue culture plate.
12. Again wash them gently with 1x PBS as done previously in step 4. Meanwhile, prepare fresh 5 mg/ml solution of sodium borohydride by dissolving it in 1x PBS. Add 500 µl of sodium borohydride per well such that the coverslips are immersed in it. Incubate under dark at room temperature for 15 min. As sodium borohydride bubbles upon dissolution, make sure that the bubbles do not settle on the coverslips by gently rocking them back and forth.
13. Wash the coverslips with 1x PBS for three times and then finally sterilize by soaking them into 70% ethanol for 10 min at room temperature. Further wash the coverslips with sterile 1x PBS and then finally add 500 µl of complete BI medium to each well and incubate at 37 °C in an incubator for an hour.
14. Coverslips are now ready to be used. If you do not want to use them immediately, sterilize them using 70% ethanol and then store them at 4 °C under dark for up to a week.

Sample preparation for the assay

1. Take a confluent tube of *Entamoeba histolytica* HM1: IMSS strain in logarithmic phase and remove the used medium. Add 10 ml of fresh complete BI medium and transfer the glass tube on ice for 5 min so as to detach the cells. The culture volume in the glass tube is 13 ml.
2. Now gently tap the tube to completely detach the trophozoites and transfer in a 15 ml conical tube. Take a small aliquot to count the number of cells using haemocytometer. Spin them down at 500 *x g* for 5 min at room temperature. Now resuspend the cell pellet in warm complete BI medium to a concentration of 40 x 10^4 cells/ml.
3. Add 0.5 ml of cells *i.e.* 2 x 10^5 cells per well over the fluorescently matrix coated coverslips and place the tissue culture plate inside a GasPak EZ pouch to maintain anaerobic conditions. Place the whole setup in 37 °C incubator.
4. Incubate for 36-48 h at 37 °C under dark.
5. Close to the end of incubation, warm an aliquot of 1x PBS and 4% PFA at 37 °C for further use.

Preparing samples for fluorescence microscopy

1. Aspirate the medium by tilting the plate to an angle without disturbing the coverslips. Now wash the cells with warm sterile 1x PBS and fix the cells immediately using 4% PFA. Incubate at room temperature for 15 min under dark.
2. After fixation, wash the sample with 1x PBS and permeabilize with 0.1% Triton-X-100 at room temperature for 10 min. Wash again and block the samples with 1% BSA in PBS for an hour at room temperature.
3. To label with Alexa 568 Phalloidin, dilute phalloidin in a ratio of 1:100 in the blocking solution (1% BSA in PBS).
4. Transfer the coverslips carefully to a humid chamber again and add 50 µl of Alexa 568 Phalloidin to each coverslip and incubate under dark at room temperature for an hour.
5. Subsequently, wash the samples by again placing them back in the tissue culture plate and adding 1x PBS.
6. After the final wash, mount the sample using mounting medium. Let the samples air dry at room temperature under dark.
7. Examine the fluorescence of the matrix and the samples using epifluorescence or confocal microscope with appropriate light source and filter sets. Since the matrix layer formed is very thin the samples should be focused accurately so that the entire field of view has uniform fluorescence. The degradation of the matrix can be observed as loss of fluorescence around the cells and would appear as a dark halo surrounding the cells.

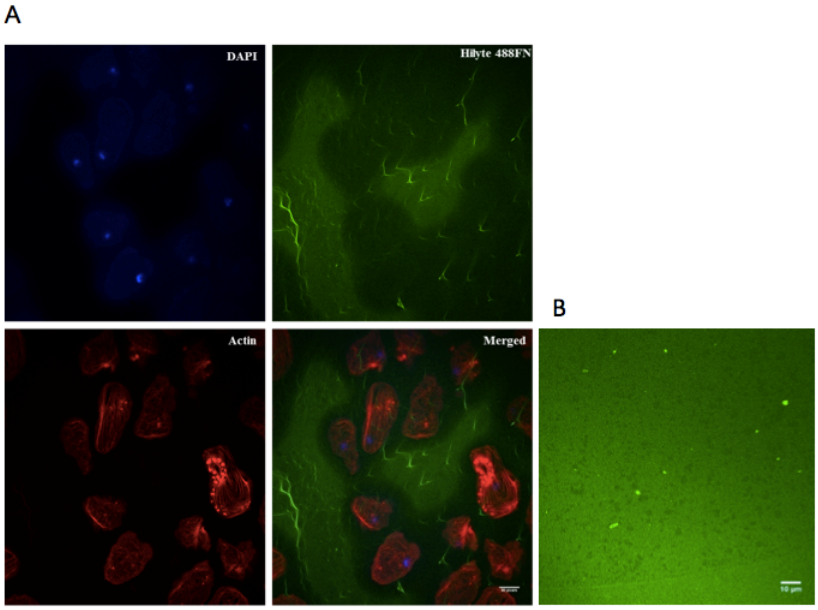

Figure 1. Rab21 mediated fibronectin degradation by amoebic trophozoites. A. Trophozoites overexpressing Rab21CA mutant were plated on a Hilyte 488 fibronectin coated glass coverslip and incubated for 48 h at 37 °C under dark. Cells were fixed, permeabilized and stained with Alexa 568 Phalloidin and DAPI. B. Undamaged Hilyte 488 Fibronectin. Scale bar, 10 µm.

Notes

1. Always prepare fresh 0.5% glutaraldehyde solution. As this solution is hazardous, avoid contact with skin and eyes.
2. Sodium borohydride should also be prepared fresh just before use. Upon dissolution, sodium borohydride releases free H_2 gas, so it should always be prepared in conical tubes with enough free space to avoid spills and bumping of the solution. Do not tightly cap the tubes containing the solution.
3. Passage your cells regularly and do not allow cells to grow to 100% confluency. Use cells which are 70-80% confluent for the experiment.

Recipes

1. 10x phosphate-buffered saline (PBS)
   Dissolve 80 g NaCl with 2 g KCl, 14.4 g Na_2HPO_4, 2.4 g KH_2PO_4
   Adjust pH to 7.4 with NaOH
   Add dH_2O to 1,000 ml
   Filter sterilize (0.2 µm)
   Stored at room temperature
2. 0.01% poly-L-lysine solution
   Dilute 0.1% solution of poly-L-lysine in sterile dH_2O in a 1:10 ratio
3. 0.5% glutaraldehyde solution
   Add 0.2 ml of stock solution to 10 ml of sterile 1x PBS
4. 5 mg/ml sodium borohydride
   Weigh 100 mg of sodium borohydride
   Dissolve in 20 ml of 1x PBS just before use
5. 4% PFA
   Weigh 4 g of PFA
   Dissolve in 100 ml of pre-warmed 1x PBS with constant stirring
6. 1% BSA in PBS
   Weigh 0.5 g of BSA and dissolve in 50 ml of 1x PBS
7. 0.1% Triton-X-100
   Make a stock solution of 10% Triton-X-100 in 1x PBS
   Add 0.5 ml of 10% Triton-X-100 in 50 ml of 1x PBS
8. 100 µg/ml Hilyte488 Fibronectin (FNR02)
   Hilyte 488 Fibronectin comes as a desiccated powder and is stored at 4 °C under dark Dissolve the powder at a final concentration of 1 mg/ml using sterile dH_2O
   Make aliquots and stored at -20 °C
9. Basal incomplete medium
   Dissolve:

3 g of Biosate peptone BBL
1 g of Glucose, 200 mg of NaCl
60 mg of KH_2PO_4
100 mg of K_2HPO_4
100 mg of L-cysteine
20 mg of L-ascorbic acid
2.28 mg of Ferric ammonium citrate in dH_2O
Adjust the pH to 6.8 using 8 N NaOH
Add dH_2O to 88 ml
Sterilized by autoclaving at 121 °C at 15 psi for 15 min

10. Complete medium
    Add 15 ml of adult bovine serum (filter sterilized) and 2 ml of 100x vitamin mixture (filter sterilized) to 88 ml of basal incomplete medium

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