Video Article

Analysis of Schwann-astrocyte Interactions Using *In Vitro* Assays

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Abstract

Schwann cells are one of the commonly used cells in repair strategies following spinal cord injuries. Schwann cells are capable of supporting axonal regeneration and sprouting by secreting growth factors [1,2] and providing growth promoting adhesion molecules [3] and extracellular matrix molecules [4]. In addition they myelinate the demyelinated axons at the site of injury [5].

However following transplantation, Schwann cells do not migrate from the site of implant and do not intermingle with the host astrocytes [6,7]. This results in formation of a sharp boundary between the Schwann cells and astrocytes, creating an obstacle for growing axons trying to exit the graft back into the host tissue proximally and distally. Astrocytes in contact with Schwann cells also undergo hypertrophy and up-regulate the inhibitory molecules [8-13].

In vitro assays have been used to model Schwann cell-astrocyte interactions and have been important in understanding the mechanism underlying the cellular behaviour.

These *in vitro* assays include boundary assay, where a co-culture is made using two different cells with each cell type occupying different territories with only a small gap separating the two cell fronts. As the cells divide and migrate, the two cellular fronts get closer to each other and finally collide. This allows the behaviour of the two cellular populations to be analyzed at the boundary. Another variation of the same technique is to mix the two cellular populations in culture and over time the two cell types segregate with Schwann cells clumped together as islands in between astrocytes together creating multiple Schwann-astrocyte boundaries.

The second assay used in studying the interaction of two cell types is the migration assay where cellular movement can be tracked on the surface of the other cell type monolayer [14,15]. This assay is commonly known as inverted coverslip assay. Schwann cells are cultured on small glass fragments and they are inverted face down onto the surface of astrocyte monolayers and migration is assessed from the edge of coverslip.

Both assays have been instrumental in studying the underlying mechanisms involved in the cellular exclusion and boundary formation. Some of the molecules identified using these techniques include N-Cadherins 15, Chondroitin Sulphate proteoglycans(CSPGs) [16,17], FGF/Heparin [18], Eph/Ephrins[19].

This article intends to describe boundary assay and migration assay in stepwise fashion and elucidate the possible technical problems that might occur.

Video Link

The video component of this article can be found at http://www.jove.com/video/2214/

Protocol

1. Boundary Assay:

1. Basic preparation: Chamber slides are coated with Poly-D-Lysine overnight and sterile glass slides are prepared before starting the experiment. Medium is prepared using Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum (FCS), and 1% Penicillin-Streptomycin-Fungizon(PSF). Also a bottle of Calcium/Magnesium free Hanks balanced salt solution (HBSS) is prepared.
2. Primary rat Schwann cells cultured in flask are trypsinized for 3 minutes using 0.1% Trypsin. Primary rat astrocyte culture is trypsinized for 5 minutes using 0.1% trypsin.

   Trypsin is inactived by adding DMEM supplemented with 10% FCS and 1% PSF.
3. Trypsinized Schwann cells and astrocytes are transferred to separate 15 mL falcon tubes and centrifuged at 300G for 5 minutes.
4. Schwann cells and astrocytes are re-suspended at the density of 2 x 10^6 (see representative results section for variations in cell density).

5. 50 µL drop of Schwann cell suspension is placed at one end of PDL coated chamber slide wells. A glass strip was used to smear the drop towards the centre of the chamber to generate a straight edge. (Using a glass cutter, a rectangular glass coverslips are cut longitudinally so that it would fit the width of chamber slides used for boundary assays)
6. A second drop of 50 µL of astrocyte suspension was placed at the opposite end of the same well and smeared towards the centre to create a straight edge parallel to the first drop smear with a small 0.2mm gap between them.
7. The chamber slides containing the drops are placed in the incubator at 37°C for 2 hours.
8. After 2 hours, cultures were washed with DMEM to remove non-attached cellular debris and fresh medium containing DMEM/ 10%FCS/ 1% PSF added.

   Note: DMEM/ 10%FCS/ 1% PSF can be supplemented with forskolin (2µM) and Bovine pituitary extract (BPE) (10µg/ mL) stimulate Schwann cell proliferation. BPE will supply the growth factors necessary to allow long term Schwann cell culture and forskolin raises the cyclic adenosine monophosphate levels within the Schwann cells to induce proliferation
9. Cells are cultured for approximately 8-10 days at 37°C , 7% CO_2 incubator. The medium requires to be changed every 3 days. The two cell fronts will eventually reach each other and a sharp boundary will form between the two cell types. At this stage experiments can be carried out analyzing factors involved in boundary formation.
10. Co-culture can be fixed using 4% formaldehyde and Schwann cells can be immunostained with anti-P75 antibody and astrocytes with anti-GFAP antibodies to identify the cells in co-culture. P75 antibody recognizes low affinity NGF receptor on Schwann cells which astrocytes lack. Anti-GFAP antibody recognizes glial firbrillary acidic protein which localizes to astrocyte cytoplasm. (Please see Figure 1A for representative boundary)

2. Migration Assay:

1. 4 well plates (15mm wells) are PDL coated overnight and prepared for creating astrocyte monolayers.
2. Primary astrocyte culture is trypsinized for 5 minutes using 0.1% trypsin. Trypsin is inactivated by adding DMEM supplemented with 10% FCS and 1% PSF and cell centrifuged at 300G for 5 minutes. (See Figure 2)
3. Astrocytes are re-suspended at $1x10^5$ cells/ mL. 1 mL of this solution is added to each PDL coated well in 4 well plates. Astrocytes are cultured for 24-48 hours until the monolayer is completely confluent.
4. To assess Schwann cell migration on astrocyte monolayers, parallel to creating astrocyte monolayers, circular PDL-coated glass coverslips are placed in a 50 mL tube and crushed using a plastic pipette to create small fragments of glass.
5. Glass fragments are transferred into a well in 6 well plate dish and using forceps suitable sized fragments are chosen and placed in other wells. 5 glass fragments are placed in each well in 6 well plates.
6. Primary Schwann cells cultured in flask are trypsinized for 3 minutes using 0.1% trypsin. Trypsinized Schwann cells are transferred to separate 15 mL falcon tube and centrifuged at 300 G for 5 minutes. Schwann cells are re-suspended at $2x\ 10^6$ cells/ mL in DMEM/10%FCS/1%PSF.
7. 20 µL droplets of Schwann cells suspension is added over each 5 coverslip fragments placed in 6 well plates and cells are incubated at 37°C , 7% CO_2 for 2 hours.
8. After 2 hours, the Schwann cells are attached to the coverslip fragments and DMEM/10% FCS/1% PSF (Supplemented with 2µM of forskolin and 10µg/ mL of BPE is added to each well so that the fragments are completely covered with medium).
9. The 6 well plates containing the fragments are then placed in the incubator for 24-48 hours until the glass fragments are fully confluent with Schwann cells.
10. Once fragments are confluent with Schwann cells, each fragment is picked up carefully using sharp forceps and dipped in HBSS to remove unattached cells and then inverted face down on to astrocyte monolayer.
11. Each well is then covered with 1 mL of DMEM/10%FCS/1%PSF and Schwann cells are allowed to migrate for 24 to 48 period. During this period experimental reagents such as growth factors can be added to assess their effect on the number and distance of migration of Schwann cells.
12. Following the migration period, the glass fragments are fixed to the bottom of each well by adding 4% paraformaldehyde (PFA) for 30 minutes. Following fixation, immunostain the Schwann cells for p75 (recognizing low affinity NGF receptor) then stain them using ABC strepavidin kit-diaminobenzidine(DAB), to allow visualization of the Schwann cells under light microscope.

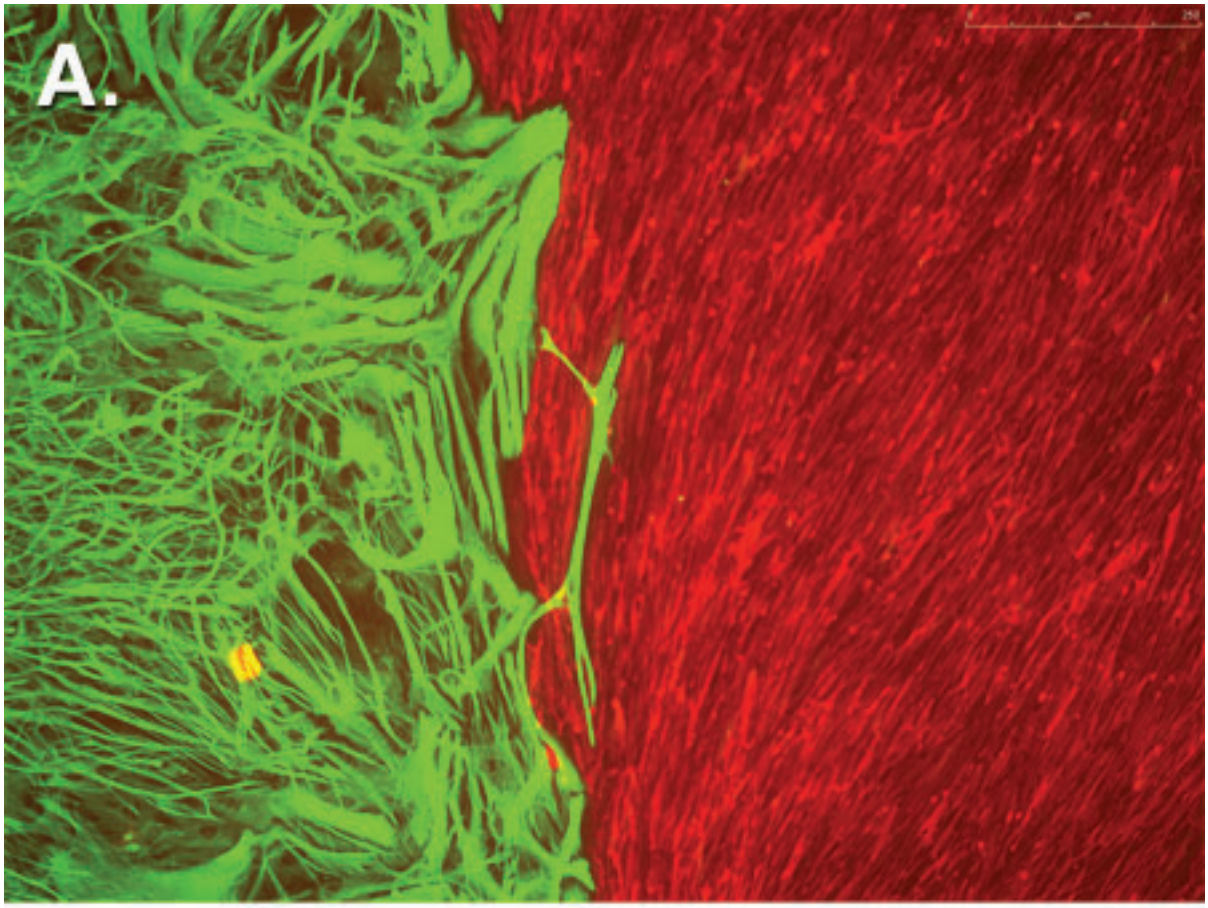
3. Representative Results:

Following 8-10 days Schwann-astrocyte co-cultures show a sharp boundary between the two cell types. This boundary is more pronounced if forskolin and BPE is used in the culture as Schwann cell proliferation is enhanced.

If no boundary is observed during the 10 day period, the time of culture can be increased further until the boundaries are established. Changing the ratio of cell density is an alternative way to increase the boundary formation. A ratio of 3:1 Schwann: astrocyte cell density can be used when preparing cell suspensions [8,18].

Following immunostaining to identify the Schwann cells and astrocytes, to assess cell intermingling a line can be drawn where the boundary is established and the number of Schwann cells migrating into the astrocyte territory can be counted under fluorescence microscope.

Below are two examples of images obtained boundaries, one with well-formed segregation of Schwann cells and astrocytes (Figure 1A) and one where the cells have not segregated well into different territories due to poor technique (Figure 1B).


A.

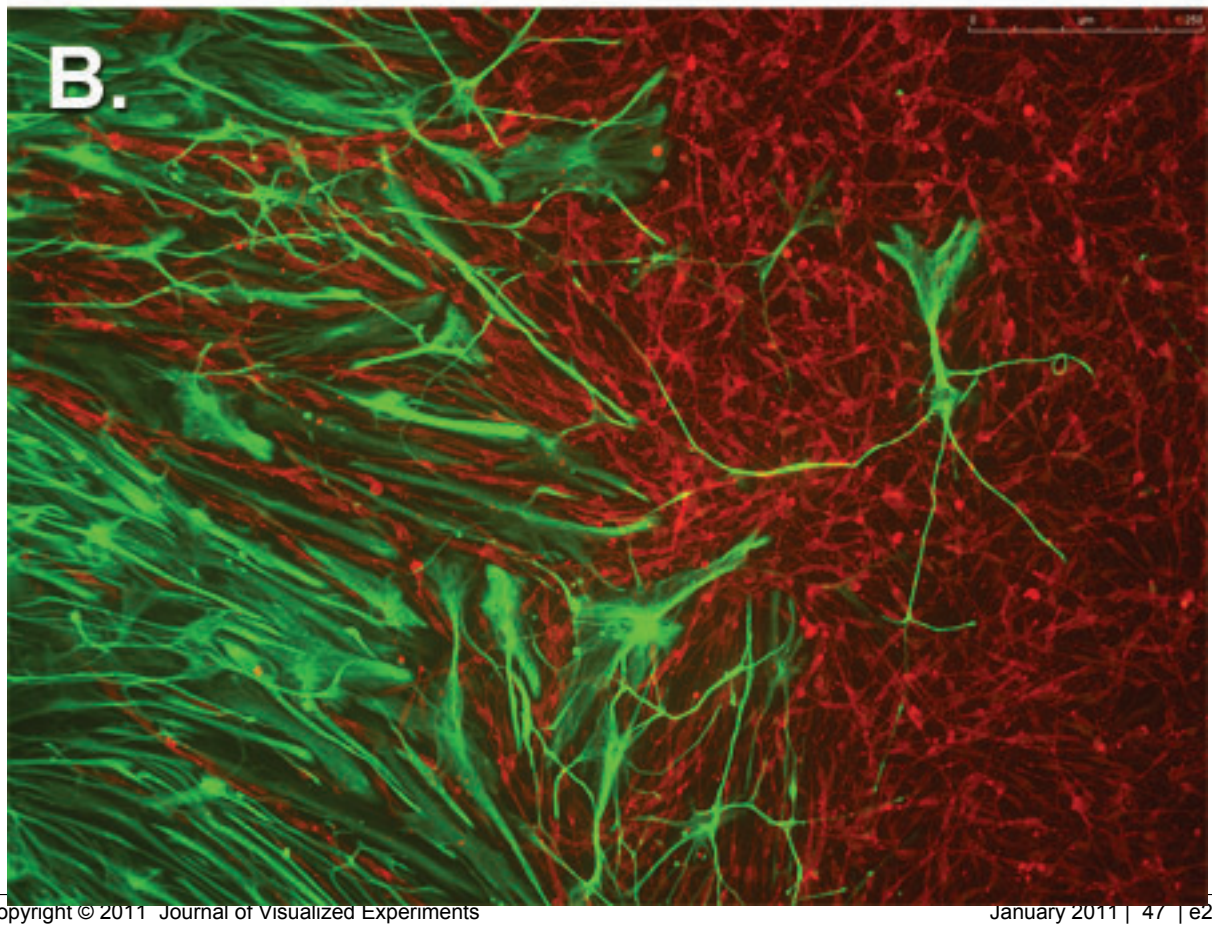

B.

Figure 1. Boundary assay: Schwann-astrocyte interaction. A) A 10 day co-culture assay in the presence of forskolin and BPE to stimulate Schwann cell proliferation demonstrates a sharp boundary between the two cell types(red=P75, green=GFAP). B) A 10 day co-culture assay where the boundary is not formed and the two cell fronts mixed. This was due to mixing of the two droplets while placing astrocyte and Schwann cell droplets next to each other during the preparation of the boundary (red=P75, green=GFAP).

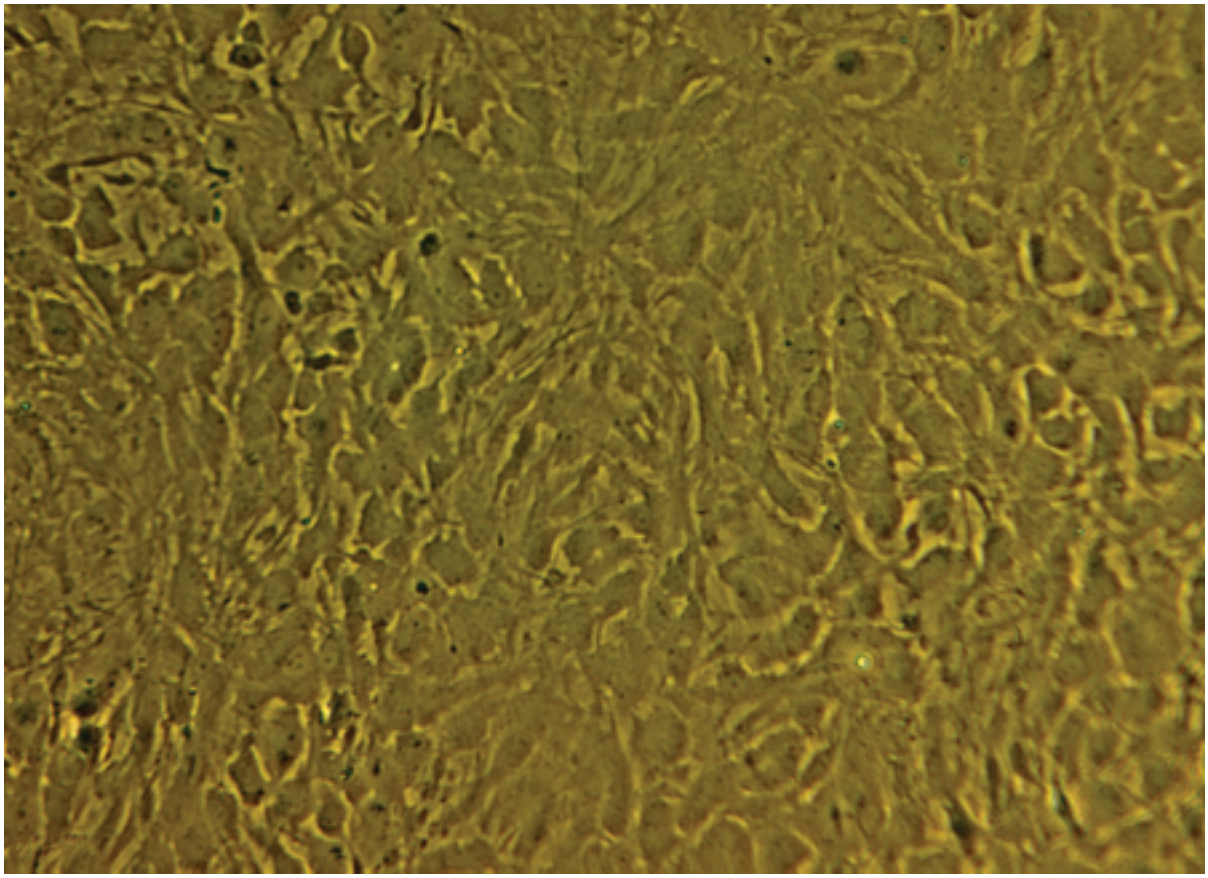


Figure 2. Representative image of confluent astrocyte monolayer

Migration assays assess movement of one cell type over the surface of another cell type and therefore assess a different phenomenon from that of boundary formation.

Practice is needed to ensure inverting the coverslips onto monolayers does not lead to damage to the Schwann cell-covered glass fragments and astrocyte monolayers by the sharp forceps. To assess the migration of Schwann cells, the maximum distance of migration and the number of migrating cells from the edge of coverslip can be measured under light microscope.

Below is the image of Schwann cells stained with P75 antibody using DAB staining on the surface of astrocyte monolayer (Figure 3).

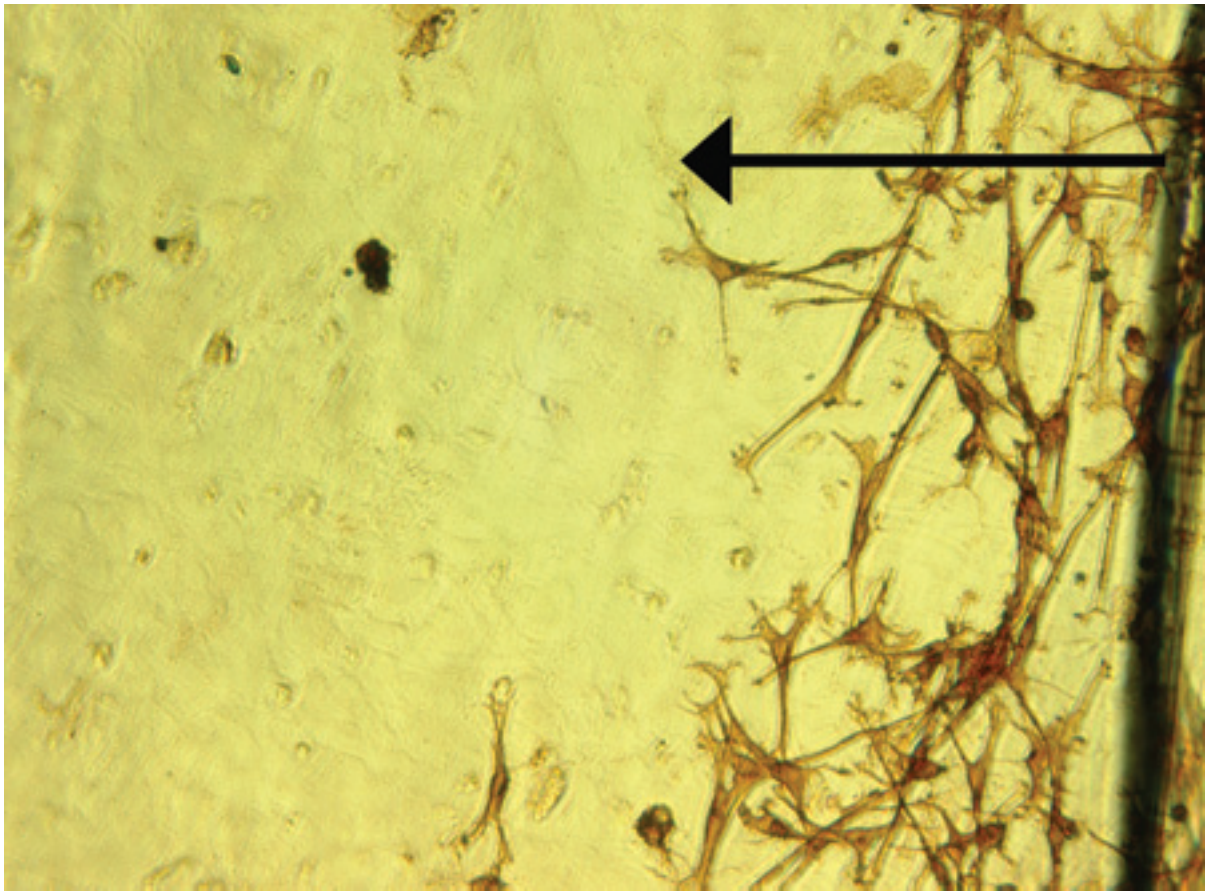


Figure 3. Migration assay using inverted coverslip assay. A) Schwann cells (brown, immunostained with p75 antibody) migrating away from the edge of coverslip. Arrow points to the direction of the cell migration away from the coverslip.

Discussion

The assays described above have been used in various studies demonstrating the role of multiple factors involved in boundary formation between the Schwann-astrocytes and limited Schwann cell migration in astrocytic environment.

Understanding the mechanisms underlying these events is essential as it would allow development of strategies to optimise and enhance the integration of Schwann cell grafts following transplantation and in doing so facilitate the exit of the regenerating axons from the graft back into the host tissue allowing formation of connections with the host tissue.

Disclosures

No conflicts of interest declared.

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