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Membrane Protein Spatial Organization in MDCK Cell Cultures

Rachel E. Neugebauer

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Abstract

Studies on the organization of membrane proteins such as GPCRs in live cells help to draw important conclusions regarding cellular communication, and have become a significant topic in drug therapy development. Observing proteins in MDCK cells, which are able to form three-dimensional cultures, provides an intermediate step to better understanding the organization and behavior of these proteins in a tissue-like culture. In this study, fluorescent stains were used on fixed cells to show the morphological differences between MDCK cells in monolayer cultures and three-dimensional cultures, which form hollow, spherical cysts of tightly packed cells. Monolayer cells were transfected to view the spatial organization of the GPCR protein, opsin, in live MDCK cells, and compared to the non-GPCR protein, plexin. Images from the transfected cells for both opsin and plexin show successful localization of the proteins to the cellular membrane in a relatively even dispersion, with no obvious sorting to any particular domain of the cell. This understanding of membrane protein organization in monolayer MDCK cultures provides a strong basis for future experiments to be performed on three-dimensional, tissue-like cultures of these cells.

Introduction

G-Protein Coupled Receptors, or GPCRs, are abundant in many human tissues and organs, and make up the largest class of membrane proteins in mammals.¹² These proteins elicit cellular response through a signal transduction pathway and are crucial to cell-signaling in many physiological processes.³ Disruptions to GPCR-dependent pathways are linked to various
chronic and life-threatening conditions such as cerebral hyperperfusion, obesity and cardiovascular disease, and they are frequently linked to tumorigenesis. Due to their variance and accessibility in tissue, GPCR proteins have become a successful molecular target for many drug therapies, including the treatment for cardiovascular disease and complications of diabetes. In fact, an estimated 30-50% of the medicine market is comprised of GPCR-related drugs. However, only a small percentage of GPCR proteins are effectively used in drug therapies, which leaves significant room for development. Understanding the organization and nature of these membrane proteins in human tissue is a necessary step in the development of therapies.

Proteins such as GPCRs in live cell membranes are commonly studied by using techniques involving fluorescence. Recent technologies involving fluorescent microscopy to study membrane proteins are valuable due to their sensitivity, accuracy, and ease of collecting measurable data in a short amount of time. These techniques can be used to measure many characteristics of GPCRs, including receptor and ligand interactions, organization and dynamics within the cell membrane. Recent experiments performed in our lab utilized PIE-FCCS to study organization and oligomerization of the GPCR protein, opsin, in cos-7 cells (derived from monkey kidney tissue). PIE-FCCS, or pulse-interleaved excitation fluorescence cross-correlation spectroscopy, uses fluctuations in the fluorescent signal caused by diffusion to enable the accurate quantification of stable protein complexes. The results from these experiments successfully indicated the concentration of opsin protein in the dimeric versus monomeric form, showing how PIE-FCCS can aid in developing a better understanding of membrane protein interactions.
However, since the cells used for this type of experiment are typically grown in flat, monolayer cultures, it can be difficult to draw specific conclusions regarding the nature of protein organization within the plasma membrane in the context of a multi-cellular structure such as an organ. The first step to making these observations on live tissue is experimentation on cells in a three dimensional culture, which will provide a closer understanding as to how these proteins will form and interact in actual living organisms. MDCK (Madin-Darby canine kidney) cells, when grown in a collagen matrix, are able to form spherical cysts composed of an outer layer of cells engulfing a hollow lumen. An intermediate step in being able to perform PIE-FCCS to study membrane protein organization in three-dimensional cultures of MDCK cells involves the study of these proteins in monolayer cultures of these cells.

This study uses commercial fluorescent dyes to display the morphological differences between MDCK cells in monolayer and three dimensional cultures. It also explores the sorting of a GPCR protein, opsin, in relation to plexin in monolayer cultures of cells. Confocal microscopy was utilized for collecting images due to the nature of these cells to form three dimensional cultures. Confocal microscopy is very useful when imaging fluorescent samples larger than about 2 µm due to its ability to improve resolution by excluding out of focus “noise” from the sample and take images in optical sections through a specimen. By observing the relative amounts of labeled protein in each “slice” of the culture, important conclusions can be drawn regarding the dispersion of both GPCR and non-GPCR proteins in the membrane. A better understanding of their natural distribution and behavior in monolayer cells will provide a basis for future experiments with PIE-FCCS and membrane proteins in tissue-like cultures.
Methods

General Procedure

Madin-Darby canine kidney cells (MDCK II) derived from cocker spaniel kidney were purchased from Sigma Aldrich and maintained according to standard protocols. Monolayer and three-dimensional cultures were fixed and stained with fluorescent commercial dyes to observe morphology. Monolayer cells were transfected with fluorescent plasmid DNA to observe membrane protein spatial organization.

Monolayer Cultures

Monolayer cultures of MDCK cells were maintained according to a protocol described by Elia and Lippencott-Schwartz.³ Cultures were plated in 100 x 22mm tissue culture dishes (Corning Inc.) with Dulbecco’s modified Eagle medium (DMEM [1X] + GLutaMAX, Life Technologies), supplemented with 10% fetal bovine serum (Life Technologies) and 1% penicillin/streptomycin (Sigma-Aldrich). Cells were split one day after reaching 100% confluency with 0.25% trypsin-EDTA (1X) (Life Technologies). Cells were passaged up to eight times, after which new cell lines were thawed from frozen stock, since highly passaged cells are not likely to form three-dimensional cultures.

Three-dimensional Cultures

Three dimensional cultures were induced similarly to standard protocol as described by Elia and Lippencott-Schwartz, but with several modifications.³ Cells were split into 100 x 22mm tissue culture dishes one day prior to plating for 3D cultures. Neutralized collagen I solution was prepared in a 15 mL test tube with the following reagents and amounts: 1.375 mL DMEM 1X
(unsupplemented), 625 µL NaHCO$_3$ (2.35 mg/mL), 125 µL Hepes (20 mM), 4.13 mL Collagen Solution from bovine (Sigma Aldrich).

The solution was mixed thoroughly by pipetting and tested on a pH strip. After confirming approximate neutrality, 100 µL collagen solution was used to coat the bottom of 35 mm glass bottom dishes to host cultures. Plates were incubated in a 37°C oven with no CO$_2$ to allow collagen to polymerize into a gel while subsequent steps were performed, or approximately two hours.

Cells were split from two plates of monolayer cultures and washed twice with DMEM. Fifty microliters of Trypan Blue Stain (0.4%, Life Technologies) was mixed with a 50 µL aliquot of cell solution, and 10 µL of the final solution was added to a counting chamber (Hausser Scientific Co.) to determine the concentration of live cells. Cells were added to the remaining Collagen I solution at a concentration of 3.0 x 10$^4$ cells/mL. The collagen-cell mixture (150 µL) was added to the coated plates, and tapped gently to ensure even distribution. The plates were incubated for one hour at 37°C with no CO$_2$, at which point a gel-like consistency was expected to form. However, the collagen matrix had only polymerized approximately 50%. Different incubation times were tested, with the optimal polymerization of approximately 70% occurring after two hours. The procedure was continued with care to not disturb the underdeveloped collagen matrix. Two milliliters of supplemented DMEM was added to the plates, and the cultures were maintained at 27°C with 5% CO$_2$. Media was changed every 3-4 days.

Approximately four days after plating, three dimensional cultures consisting of small, tightly packed cells in a spherical shape appeared to form within the loose collagen matrix. Extra care
was taken when changing media so as not to disturb the matrix and cultures. Fixation, staining, and imaging were performed 2-3 days after the formation of three dimensional cultures.

**Fixation & Staining**

Cultures of MDCK cells in both monolayer and 3D culture form were fixed and stained in order to image their morphology. For monolayer cultures, cells were plated onto 35 mm glass bottom dishes (No. 1.0, MatTek Co.) in DMEM and allowed to reach 75-95% confluence prior to fixation and staining. Three dimensional cultures were fixed and stained on the same glass bottom dishes in which they were induced. To prepare for fixation, media was removed from each dish. Cultures were washed twice with 1X PBS buffer (phosphate buffered saline, Life Technologies). To fix cells, 300 µL of 4% formaldehyde (Sigma Aldrich) in PBS were added to the cultures and incubated at room temperature for 15 minutes. Two more washes with 1X PBS were performed to remove fixative. Extra care was taken when working with 3D cultures in order to avoid disturbance to the loose collagen matrix.

Labeling solution I was prepared, containing Alexa Fluor 594 wheat germ agglutinin (WGA) and Hoechst 33342 dye (Molecular Probes, Life Technologies), which target the cell membrane and nucleus, respectively. Alexa Fluor-WGA stock (1.0mg/mL) was diluted to 5.0 µg/ml and Hoechst stock (1.0mM) to 2 µM into a single staining solution in PBS, for a final volume of 4 mL. Three hundred microliters of the staining solution were added to each culture, and the plates were incubated at room temperature for 10 minutes. After removing staining solution, two more PBS washes were done.

Labeling solution II, containing Alexa Fluor 488 Phalloidin actin stain (Molecular Probes, Life Technologies), was prepared by diluting the stock solution (6.6 µM) to 2.5% volume per total
solution volume in PBS. BSA (albumin from bovine serum, Sigma Aldrich) was added to the solution at a volume of 1% of the total solution volume, as recommended by the dye supplier, to avoid non-specific background staining in the culture. Three hundred microliters of staining solution II were applied to each culture, and plates were incubated for 20 minutes at room temperature. After removing staining solution, cultures were washed twice with PBS. To prepare for imaging, 2 mL PBS buffer was added to each plate. Care was taken not to expose stained cultures to excessive light before imaging, as dyes are photo-sensitive.

**Transfection of Monolayer Cultures**

Cells were transfected in order to observe membrane protein spatial organization in monolayer cultures. MDCK cells were split into 35 mm glass bottom dishes. Upon reaching 70-90% confluency, the cells were transfected with Lipofectamine 2000 transfection reagent (Life Technologies), according to standard protocol as described by the supplier. Cells were transfected with the GPCR protein, opsin, or plexin to compare relative protein dispersion. Opsin-EGFP/ mCherry plasmids and plexin-EGFP/ mCherry plasmids were produced for previous experiments from mouse opsin and PlexinA4 cDNA and their protocols have been described in detail.⁷,¹⁰

**Imaging**

Images of stained and transfected cell cultures were taken on a Nikon A1plus confocal laser scanning microscope equipped with a Nikon 1.4NA, 100x Plan Apo objective. Images were processed using the Nikon Elements software package. Stained cultures were observed with a pinhole radius of 37.04 µm. Hoechst 33258 dye (nuclear stain, blue) was viewed at an emission wavelength of 450.0 nm and an excitation wavelength of 405.0 nm. Alexa Fluor 488 (actin stain,
green) was imaged at an emission wavelength of 525.0 and an excitation wavelength of 488 nm, and Alexa Fluor 594 (membrane probe, red) was imaged at an emission wavelength of 595.0 nm and an excitation wavelength of 561.0 nm.

For transfected cultures, the pinhole radius was set to 65.13 µm. EGFP-labeled opsin and plexin were imaged at an emission wavelength of 525.0 nm and an excitation wavelength of 488.0 nm, while mCherry labeled protein was imaged at emission wavelength 595.0 nm and excitation wavelength 561.0 nm.

**Results & Discussion**

**MDCK monolayer cell culture morphology**

A typical section of stained monolayer culture of MDCK cells were imaged using the equipment described above. Twenty consecutive images were taken starting from the bottom of visible culture (2724.15 µm) and moving upward in 0.699 µm slices until reaching the top of the culture (2738.15 µm). Images A (slice 4 of 20) and B (slice 7 of 20), shown below, display single slice images taken from the series (For entire series of images from stained monolayer culture, see appendix B1).
Figure 1. Fixed MDCK cells in monolayer culture were stained with commercial dyes in order to show morphology in flat culture. Images A & B (above) display a typical section of fixed MDCK cells in monolayer culture after staining, taken at 2.096 µm and 4.192 µm from the bottom of visible culture. Alexa Fluor 594 wheat germ agglutinin (red) and Hoechst 33342 dye (blue) were used to stain membranes and nuclei, respectively. Alexa Fluor 488 Phalloidin actin stain (green) highlights the cytoskeleton.

Image A was taken 2.096 µm from the bottom of visible culture. The Alexa Fluor 488 Phalloidin actin stain (shown in green) highlights the cytoskeletal framework through the bottom side of the cell culture. Image B was taken 4.192 µm from the bottom of visible culture. In addition to the aforementioned actin stain, Hoechst 3342 dye highlights the membrane in blue, and Alexa Fluor 594 stains the membrane in red. From this image, the overall morphology of MDCK cells in monolayer culture is visible. Large cells are adherent to the bottom of the culture plate and pack tightly against each other in a single, flat layer. This type of culture is a
typical target for experiments involving membrane proteins, such as PIE-FCCS, but may not accurately portray how membrane proteins would behave in actual live tissue.

**MDCK three-dimensional cell culture morphology**

Stained three-dimensional cultures of MDCK cells grown in a collagen matrix were imaged in a similar manner to monolayer cultures. Twenty five consecutive images were taken starting from the bottom of visible culture (2676.18 µm) and moving upward in 1.404 µm slices until reaching the top of culture (2710.03 µm). Figure 2, below, shows a three-dimensional model of the lower half of a typical three-dimensional culture, derived from all 25 images. Due to limitations of the microscope and the higher location of the cells within the collagen matrix, the image quality was not as high as for the monolayer culture. However, images displaying the bottom half of the spherical culture provide insight to its overall morphology.
Figure 2. Fixed MDCK cells in three dimensional culture within a collagen matrix were stained with commercial
dyes in order to show morphology. The image above displays the bottom half of a typical three dimensional
culture after staining. Alexa Fluor 594 wheat germ agglutinin (red) and Hoechst 33342 dye (blue) were used to
stain membranes and nuclei, respectively. Alexa Fluor 488 Phalloidin actin stain (green) highlights the
cytoskeleton.

The three-dimensional culture of MDCK cells appears as a large sphere of smaller and more
tightly packed cells than in monolayer culture. The actin stain, nuclear probe and membrane
probe are each visible in the outer later of the culture, and it appears that the collagen matrix
trapped small amounts of dye, as seen in the red clouds surrounding the culture, and the slight
blue “noise” in the image. Figures 3-5 (below) display cross-sections from the three-
dimensional culture taken from images 8, 14 and 22 of 25 from the series (For entire series of
25 images, see Appendix B2).
Figures 3-5. Fixed MDCK cells in three dimensional culture within a collagen matrix were stained with commercial dyes in order to show morphology. The figures above display cross-section views of a typical three dimensional culture after staining. Alexa Fluor 594 wheat germ agglutinin (red) and Hoechst 33342 dye (blue) were used to stain membranes and nuclei, respectively. Alexa Fluor 488 Phalloidin actin stain (green) highlights the cytoskeleton. The main images show the cross-section from a top view of the culture, while the images to the bottom and right indicate the location of the slice from side views of the three dimensional cultures.

Figures 3-5 provide a clearer understanding to the overall morphology of the three-dimensional culture. Figure 3 shows a cross section taken 9.829 µm from the bottom of visible culture, and displays a layer of cells at the the bottom of the spherical culture, which form a
round shape as expected from the bottom of the cyst. The cells resemble those from the monolayer culture, but appear to be smaller and much more tightly packed.

Figure 4, taken 18.254 µm from the bottom of visible culture, helps to explain why there appears to be a high fluorescent intensity towards the bottom of the three-dimensional culture view in Figure 2 (above). This image was taken very near to the bottom of cell culture, where the formation of a hollow lumen is visible, but is still surrounded by multiple layers of cells due to its developmental stage. When MDCK cells are plated in a collagen matrix, they proliferate and grow into a spherical shape while the cells toward the middle of the cyst undergo apoptosis until a hollow lumen is formed, surrounded by a single layer of cells. The thickness of cells toward the bottom of the culture indicates that the bottom layer of the cyst is in a late stage of development, and should eventually resemble the single-cell layer cyst shown in figure 5.

Figure 5, taken at 29.487 µm and near the center of the overall cyst, reveals that the largest part of the spherical culture consists of a single layer of tightly packed cells surrounding a hollow lumen. This structure suggests that the middle section of the cyst is fully developed. This culture model would provide a suitable host for experiments with membrane proteins as an intermediate step for understanding how the proteins of interest would behave in live tissue. Observing these proteins in MDCK cells from monolayer cultures provides a strong base to help design further experiments on three-dimensional cultures.
Opsin-transfected Cells

Monolayer cultures of opsin-transfected MDCK cells were imaged in 22 consecutive 0.500 µm slices starting from the bottom of visible culture (2671.85 µm) and ending at the top of visible culture (2682.38 µm). Figure 6, (slice 7 of 22) displays a single cross section image from the series (for entire series of images, refer to appendix B3).

Figure 6. The image above shows the distribution of the GPCR protein, opsin within a typical MDCK cell in monolayer culture approximately 24 hours after transfecting with opsin mCherry/ eGFP plasmid DNA. MCherry plasmid is shown in red while eGFP is shown in green. The main images shows the top view of a cross section from the cell, while the images to the bottom and right indicate the location of the image from side views of the cell.
Figure 6 shows the distribution of the GPCR protein, opsin, in a transfected MDCK cell in monolayer culture. The panels to the bottom and right of the main image show the location from where the slice was taken on three-dimensional models of the cell built from the series of images. The main cross-section image of the transfected cell displays successful localization of opsin in the cell membrane, which is evident by the intensity of the green signal from opsin-EGFP outlining the cell. It shows even distribution of the membrane protein through all sides of the cell. The side views of the three-dimensional cell indicate that opsin is distributed evenly throughout the cell, rather than being sorted into different domains, which is often associated with the presence of tight junctions in cell cultures.

**Plexin-transfected MDCK Cells**

Monolayer cultures of plexin-transfected MDCK cells were imaged in 24 consecutive 0.500 µm slices starting from the bottom of visible culture (2654.83 µm) and ending at the top of visible culture (2666.38 µm). Figure 7, (slice 10 of 24) displays a single cross-section image from the series (for entire series of images, refer to appendix B4).
Figure 7. The image above shows the distribution of the non-GPCR membrane protein, plexin within an MDCK cell approximately 24 hours after transfecting with plexin mCherry/ eGFP plasmid DNA. mCherry plasmid is shown in red while eGFP is shown in green. The main images shows the top view of a cross section from the cell, while the images to the bottom and right indicate the location of the image from side views of the cell.

Figure 7 displays the dispersion of plexin through an MDCK cell from monolayer culture. The fluorescent signal is slightly weaker than from opsin, which could be improved by increasing the
amount of plasmid used during transfection. However, there is still sufficient fluorescent signal from EGFP labeled protein to visibly mark the membrane. As with the case of opsin, the cross-section image indicates a strong, successful localization of the plexin protein to the plasma membrane, and a rather even distribution throughout the sides of the cell. Similarly, the outside panels displaying the three-dimensional model of the cell indicates even organization of plexin throughout the membrane, as opposed to being sorted into any specific domains.

**Conclusion**

Madin-Darby canine kidney cells grown in monolayer and three-dimensional cultures were fixed and dyed with commercial stains in order to compare culture morphology. Imaging of monolayer cultures using confocal microscopy showed large, tightly packed cells adherent to the plate, while three-dimensional culture techniques yielded large, hollow, spherical cysts of very tightly packed, small cells. Live cells in monolayer culture were transfected with opsin and plexin to gain a better understanding of the spatial organization and sorting of GPCR versus non-GPCR proteins in live MDCK cells. Images indicated successful transfection and localization of both types of protein to the cell membrane. Both opsin and plexin appeared to be evenly distributed throughout the cell membrane in every direction, with no evidence of sorting to particular domains.

These observations provide better insight into the organization of membrane proteins in MDCK cells and will help to design further studies that observe the behavior of these proteins in three-dimensional cultures. Future experiments will involve stable transfections in MDCK cells which will then be plated in three-dimensional cultures expressing the proteins of interest. PIE-FCCS will then be used to observe protein interactions and oligomerization in the cell.
membrane. Understanding the behavior of membrane proteins in three-dimensional mammalian cell cultures will provide deeper insight to how these proteins might behave in actual live tissue, which would significantly aid in drug development and other therapies targeting membrane proteins.

**Acronyms Used:**

MDCK: Madin Darby canine kidney

GPCR: G-Coupled protein receptor

PIE-FCCS: pulse-interleaved excitation fluorescence cross-correlation

DMEM: Dulbecco’s modified Eagle medium

GFP: Green fluorescent protein

WGA: Wheat germ agglutinin
References


9. Paddock, S. W.; Fellers, T. J.; Davidson, M. W. *Nikon MicroscopyU*. 


Appendix A: Safety Considerations

**General Laboratory Safety Precautions:** In order to avoid contact with any reagents that may cause harm, general precautions were taken when working in the lab. Protective, lightweight clothing and closed toed shoes were worn at all times. Gloves were worn when working with reagents, which were handled inside an appropriate hood. All materials were disposed of in proper containers.

**Laboratory Equipment:** Thorough training was completed before working with laboratory equipment to ensure safe and proper use. Work was done under supervision of a graduate student or professor at all times.

**Laser Lab Safety:** General lab precautions were used when working in the laser lab. All work was done with an experienced graduate student or instructor. Special care was taken to avoid eye exposure to lasers, which can cause serious damage.

**Specific Safety Considerations (by reagent):** Material Safety Data Sheets provided by suppliers of each reagent were referred to for safety information. Specific safety considerations for any reagents that may cause serious physical harm are described below:

- **Hoechst 33342 Dye** – Hoechst dye may cause serious eye damage upon contact. Skin contact should be avoided. Gloves and protective eyewear were worn at all times when handling dye, and hands were washed thoroughly after use.

- **Alexa Fluor 488 Phalloidin** – Phallotoxin is toxic upon skin contact and harmful if inhaled or swallowed. Gloves and protective eyewear were used, and dye was handled in the hood.
**Trypan Blue** – This stain has possible carcinogenic effects and reproductive toxicity. Gloves were worn at all times, always used in cell culture hood, and disposed of properly.

**Formaldehyde** – Hazards: combustible, toxic if swallowed, in contact with skin or inhaled, causes severe skin burns and eye damage, may cause genetic defects, may cause cancer, causes organ damage, and harmful to aquatic life. This reagent was handled with extreme care under a hood at all times. Gloves and long sleeves were worn to avoid skin contact, and protective eyewear was utilized. Waste was disposed of carefully in the proper waste container.

**Pen-Strep** - May cause skin and eye irritation upon contact, and respiratory irritation and asthma symptoms if inhaled. Reagent was used under hood at all times, with protective clothing, gloves and eyewear.
Appendix B1: Image Series from Stained MDCK Cells in Monolayer Culture

Appendix B1 shows the 20-image series taken of fixed and stained MDCK cells in monolayer culture. Images were taken in .699 µm slices starting from the bottom of visible culture and ending at the top. Alexa Fluor 594 wheat germ agglutinin (red) and Hoechst 33342 dye (blue) were used to stain membranes and nuclei, respectively. Alexa Fluor 488 Phalloidin actin stain (green) highlights the cytoskeleton.
Appendix B2 shows the 25-image series taken of fixed and stained MDCK cells in three-dimensional culture. Images were taken in 1.4 μm slices starting from the bottom of visible culture and ending at the top. Alexa Fluor 594 wheat germ agglutinin (red) and Hoechst 33342 dye (blue) were used to stain membranes and nuclei, respectively. Alexa Fluor 488 Phalloidin actin stain (green) highlights the cytoskeleton.
Appendix B3: Image Series from Opsin-transfected MDCK Cells in Monolayer Culture

Appendix B3 shows the 22-image series taken of opsin-transfected MDCK cells from monolayer culture. Images were taken approximately 24 hours post-transfection in 0.5 μm slices starting from the bottom of visible culture and ending at the top. MCherry plasmid is shown in red while eGFP is shown in green.
Appendix B4 shows the 24-image series taken of plexin-transfected MDCK cells from monolayer culture. Images were taken approximately 24 hours post-transfection in 0.5 µm slices starting from the bottom of visible culture and ending at the top. MCherry plasmid is shown in red while eGFP is shown in green.