

Size-selective assessment of tight junction paracellular permeability using fluorescently labelled dextrans

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- Tight junctions encircle epithelial and endothelial cells
- Tight junctions restrict paracellular diffusion of ions and non-ionic tracers depending on charge and size
- Paracellular permeability of 4 kDa FITC Dextran and 70 kDa Rhodamine B Dextran is determined with the FLUOstar OPTIMA

Introduction

Epithelial and endothelial cells are joined to each other via a set of intercellular junctions that are important for tissue formation and function. Tight junctions are composed of transmembrane components that mediate adhesion and form the paracellular diffusion barrier. These proteins interact with a cytoplasmic plaque composed of adaptor proteins that recruit various signalling components and interact with the actin cytoskeleton¹. In epithelia, tight junctions are the most apical intercellular junction, forming a continuous structure that completely circumvents the cell².

In endothelia, the position of tight junctions can vary, but their composition and function are similar to tight junctions in epithelia³. Epithelial tight junctions create a functional boundary between the apical and basolateral cell surface domains, thereby regulating diffusion along the paracellular pathway². Tight junctions form a fence that prevents the intermixing of lipids in the outer leaflet of the plasma membrane⁴. They also form a semipermeable paracellular gate that restricts diffusion in a charge- and size-selective manner⁵. The ion- and size-selectivity of the paracellular pathway differs among epithelia and is regulated by different physiological and pathological stimuli. The main structural components of the paracellular gate are thought to be the claudins, a family of transmembrane proteins responsible for ion-selectivity. Another transmembrane protein, occludin, is thought to regulate the diffusion of small hydrophilic tracers along a size-selective pathway.

Since the molecular mechanisms of size-selective diffusion are poorly understood, studying barrier formation by endothelia and epithelia is important, especially in treating such diseases as chronic inflammations or cancer. Furthermore, such knowledge could be used to manipulate the paracellular permeability of drugs to enhance their uptake into protected organs such as the brain⁴. Here we describe a scalable, easy to use, and reliable tight junction permeability assay that analyzes tight junction integrity, as well as the rates of size-selective paracellular permeability.

Materials and Methods

All materials were obtained through normal distribution channels from the manufacturer stated.

- MCF-10A-95 cells and MCF-10-JB cells were cultured as described⁵
- 12-well clusters of tissue culture inserts in polystyrene plates were from Corning (catalogue no 3401). Culture systems from 6 to 96 wells are commercially available.
- Fluorescently labelled dextran and rhodamine-labelled dextran were from Sigma (4kD FITC-Dextran, FD-4; 70kD Rhodamine B-Dextran, R-9379)

Instruments

- EVOM voltmeter, World Precision Instruments, Sarasota, FL., USA for TER (transepithelial electrical resistance) measurements
- FLUOstar OPTIMA, BMG LABTECH, Offenburg, Germany for fluorescence measurements

General considerations

Many of the commonly used methods to analyze the tight junctions are based on cultured epithelial cells since they easily allow quantification of junctional properties. Furthermore, studies are generally performed with epithelial cells cultured on permeable supports since they allow easy measurements of electric currents or tracer flux across monolayers. Epithelial cells grown on such permeable supports often polarize and differentiate more extensively.

Investigation of tight junctions often involve transfection of wild type or mutant tight junction proteins; therefore, it is important that such cell lines express the transfected proteins in a homogenous manner. This is generally achieved by cloning or using bi-cistronic expression vectors. Furthermore, when a new cell line is to be used, pilot studies with non-transfected cells should be done to determine how quickly the cell line forms mature monolayers that exhibit stable functional junctions. Analysis of cell lines, whose transepithelial electrical resistance and paracellular permeability do not reach stable values within a few days, can be very unreliable since the observed differences may just reflect variations in junction formation, maturation and differentiation.

Analysis of the paracellular gate

Tight junctions restrict paracellular diffusion of ions and hydrophilic non-ionic tracers in a selective manner, differentiating by charge and size. The selectivity of the paracellular barrier varies from one epithelial tissue to another and different stimuli or manipulations can result in opposite effects on the permeability of ions and tracers. Hence, the determination of only ion or hydrophilic tracer permeability and not both, often does not completely characterize the functional state of the paracellular diffusion barrier. Ion permeability of tight junctions is generally determined by measuring transepithelial electrical resistance (TER). Paracellular permeability of hydrophilic tracers can be monitored with compounds that are labelled fluorescently, such as dextrans. Herein, fluorescently labelled dextrans (4kD FITC dextran and 70 kD Rhodamine dextrans) are used as tracers since different sizes can be analysed by the same detection method; moreover, the use of both FITC and Rhodamine labelled dextrans allows for the analysis of two different tracers in the same culture.

Experiments

Cells are plated to confluence in 12-well tissue culture inserts and are left for at least 5 days to form differentiated monolayers, with the medium being replaced every second day. The analysis is started by measuring TER for later comparison with the paracellular diffusion results. The medium is then replaced with fresh tissue culture medium, adding 1 ml to the outer and 250 µl to the inner chamber of 12-well tissue culture inserts. The cultures are then left to equilibrate in the tissue culture incubator for at least 30 minutes. Then 50 µl of the fluorescent tracer(s) is added into the inner chamber, bringing the total volume to 300 µl, so that the final concentration of tracer(s) is between 1 to 3 mg/ml. The cultures are then incubated at 37°C for 3 to 4 hours.

The filters are removed and the diffused fluorescent tracer is measured by the FLUOstar OPTIMA (FITC-Dextran: Exc: 485 nm and Em: 544 nm and/or Rhodamine B-Dextran: Exc: 520 nm and Em: 590 nm). The amount of diffused dextran can be determined using calibration curves established just with the stock solution, and kinetic experiments can be performed by removing small samples of the outer chamber medium every hour.

Results

Neither TER nor tracer permeability measurements alone reflect the paracellular pathway properties, but both are a composite of the paracellular and transcellular route. For example, an increase in the 4kD-dextran permeability could be due to either increased paracellular diffusion and/or increased rates of fluid-phase transcytosis. That means that transcytosis needs to be considered.

Figure 1A shows the results of TER measurements from two different cell strains, indicating that only MCF10A-95 cells form electrically tight monolayers.

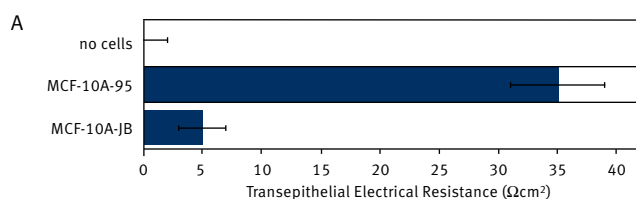


Fig. 1A: Analysis of the paracellular gate in two different strains of MCF-10A cells. MCF-10A-95 and MCF-10-JB cells were cultured on permeable tissue culture supports for 5 days and then analysed by measuring transepithelial electrical resistance

Paracellular permeability of 4kD FITC Dextran and 70 kD Rhodamine Dextran was determined by adding a mix of the two tracers to the apical chambers of the cultures. Figure 1B shows that both cell lines reduced the amount of paracellular flux of both types of dextran.

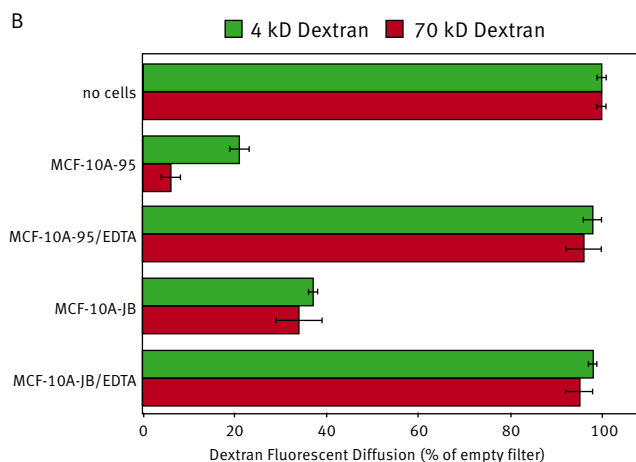


Fig. 1B: Paracellular permeability of cultures incubated with dextran in the absence or presence of EDTA, which dissociates cell-cell junctions, was compared with diffusion across empty filters. Note, only the MCF-10A-95 strain develops a electrically resistant barrier that restricts paracellular flux by size. (Modified from Sourisseau et al., Mol. Cell Biol. 26, 2387-2398, 2006).

This was calcium-dependent, suggesting that the formation of cell-cell junctions is important for barrier formation. The data shows that MCF-10A-95 cells not only form more efficient barriers than MCF-10A-JB, but they also restrict diffusion in a size-selective manner, supporting the conclusion that they form functional tight junctions. Thus, MCF10A-95 cells are able to form a functional epithelial barrier that restricts paracellular diffusion in an ion- as well as size-selective manner. These results further illustrate that assessment of the paracellular barrier formation requires the analysis of both TER and paracellular tracer diffusion.

Conclusion

When analyzing tight junctions, it is required to test for expression/localization of protein markers as well as to assay for paracellular gate function. The junctional paracellular gate exhibits complex features; hence, a single assay does not allow a meaningful conclusion. Minimal analysis requires the determination of ion selectivity (TER) and size-selective tracer diffusion.

We describe here a method for the quick and reliable analysis of size-selective paracellular tracer diffusion using fluorescent dextrans of different molecular weights. The assay is robust and allows for the analysis of many different samples in parallel; thereby, facilitating the analysis of different clonal lines in structure/function projects or the determination of regulatory pathways. As epithelial cells can also be grown in 96-well plate inserts, the assay could easily adapted to large scale screens to identify genome-wide regulatory pathways or small molecules to modify junctional permeability.



Fig. 2: BMG LABTECH's multimode microplate reader FLUOstar OPTIMA

Using the FLUOstar OPTIMA (Figure 2) for these experiments allows quick and consistent fluorescence measurements in either kinetic or endpoint format. As a multimode detection reader the FLUOstar OPTIMA is also able to perform time-resolved fluorescence, absorbance and luminescence.

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