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RESEARCH PAPER

Thrifty, Rapid Intestinal Monolayers (TRIM) Using Caco-2 Epithelial Cells for Oral Drug Delivery Experiments

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ABSTRACT

Purpose Caco-2 monolayers are the most common model of the intestinal epithelium and are critical to the development of oral drug delivery strategies and gastrointestinal disease treatments. However, current monolayer systems are cost- and/or time-intensive, hampering progress. This study evaluates two separate methods to reduce resource input: FB Essence as a fetal bovine serum (FBS) alternative and a new, 3-day Caco-2 system deemed "thrifty, rapid intestinal monolayers" (TRIM).

Methods Caco-2 cells were cultured with FB Essence and compared to cells in 10% FBS for proliferation and monolayer formation. TRIM were compared to commonly-used 21-day and Corning® HTS monolayer systems, as well as mouse intestines, for permeability behavior, epithelial gene expression, and tight junction arrangement.

Results No amount of FB Essence maintained Caco-2 cells beyond 10 passages. In contrast, TRIM compared favorably in permeability and gene expression to intestinal tissues. Furthermore, TRIM cost $109 and required 1.3 h of time per 24-well plate, compared to $164 and 3.7 h for 21-day monolayers, and $340 plus 1.0 h for the HTS system.

Conclusions TRIM offer a new approach to generating Caco-2 monolayers that resemble the intestinal epithelium. They are anticipated to accelerate the pace of in vitro intestinal experiments while easing financial burden.

KEY WORDS Caco-2 · in vitro model · oral delivery · permeability · tight junctions

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ABBREVIATIONS

BIEDE	Biocoat® Intestinal Epithelium Differentiation Environment
CLDN1	Claudin 1
DMEM	Dulbecco's Modified Eagles Medium
FBE	FBEssence
FBS	Fetal bovine serum
FITC-DX4	4 kDa molecular weight dextran, tagged with fluorescein isothiocyanate
HTS	Corning® Biocoat® HTS monolayer system
P##	Passage number ## of Caco-2 cells
PPZ	1-phenylpiperazine
qPCR	Quantitative polymerase chain reaction
Rhod123	Rhodamine 123
SLS	Sodium lauryl sulfate
TEER	Transepithelial electrical resistance
TRIM	Thrifty, rapid intestinal monolayers
ZO-1	Zonula occludens 1

INTRODUCTION

The unique ability of the Caco-2 cell line to differentiate into enterocytes was first reported in 1983 (1), and it has since become the most commonly-used model of the small intestinal epithelium (2,3). Monolayers comprising differentiated Caco-2 cells are used by labs around the globe to aid research in oral drug delivery (4–6), gastrointestinal disease (7–10), safety testing (11–13), and more. To achieve differentiation, Caco-2 cells are cultured on porous membranes with separate media chambers feeding both the apical (top) and basal (bottom) surfaces of the cell monolayer (Fig. 1a). These monolayers generally correlate well to human small intestines with regard to molecular permeability and gene expression (14), and they develop the same type of tight junction complexes as human intestinal enterocytes (15,16). However, the majority of available Caco-2 systems are expensive and/or labor intensive to

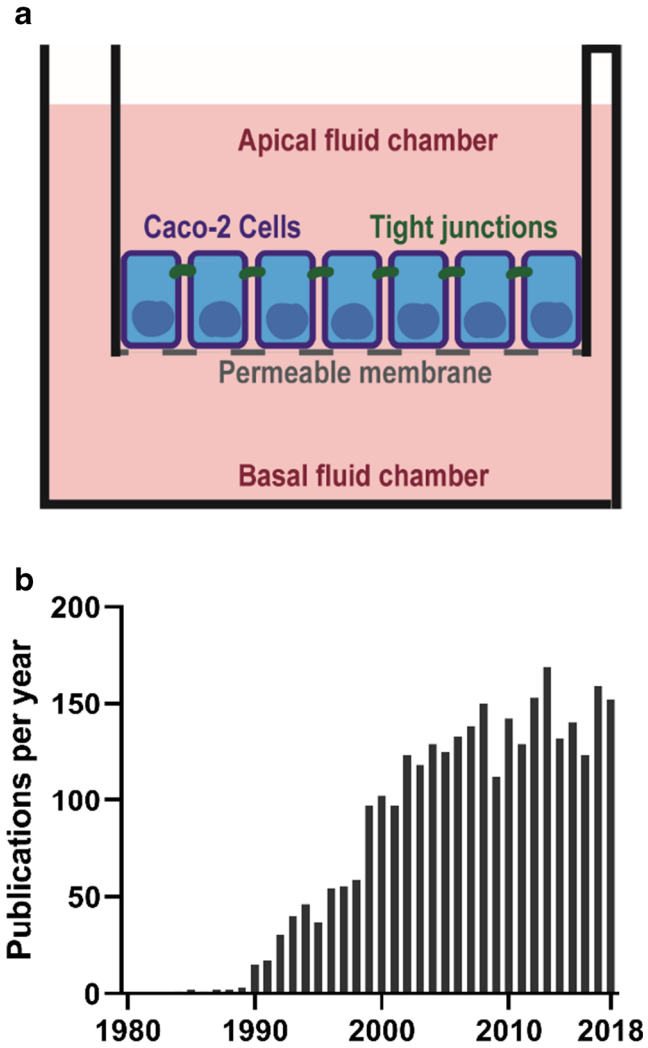

Fig. 1 Caco-2 monolayers are the most common model of the intestinal epithelium. (**a**) Caco-2 cells are seeded on a permeable membrane between two fluid chambers and differentiate into a single epithelial layer. (**b**) Medline Trends data for "Caco-2 Monolayers." Accessed March 2019

develop. With hundreds of new publications relying on these cells every year (17) (Fig. 1b), there is a major need to streamline the monolayer assay for more efficient intestinal research.

Traditionally, Caco-2 monolayers are seeded and maintained for 21 days to allow full differentiation and tight junction formation (2,18). These monolayers are well-established and highly characterized. However, the tri-weekly media changes are tedious and often lead to bacterial or fungal infection in the cultures. To address this, Corning® introduced the Biocoat® Intestinal Epithelium Differentiation Environment (BIEDE) in 1997, enabling Caco-2 monolayers to differentiate in only three days while offering similar permeability to 21-day monolayers (19). However, the BIEDE system and its current iteration, called Biocoat® HTS monolayers, cost approximately $370 per 24-well plate as of June 2019. This figure is almost $200 per plate more than 21-day monolayers, potentially limiting the productivity of labs that depend on them for their experiments. As a result, in the approximately two decades since, several labs have developed and characterized their own monolayer systems (20–23). However, it remains unclear how the systems compare to the traditional monolayers for different assay types (e.g. gene expression vs. permeability) and final costs.

Here, we present two possible strategies for reducing costs and time input for Caco-2 research and examine their effects on the resulting monolayers. First, we evaluate the fetal bovine serum (FBS) alternative FB Essence (FBE) for reduction of cell line maintenance expenses. 10% FBS has long been the standard serum addition to Dulbecco's Modified Eagles Medium (DMEM) for Caco-2 cell culture media (24), but its use incurs high costs, composition uncertainty, and ethical unease regarding the fetal calf sourcing (25). FB Essence is a less costly alternative, derived from adult bovine blood and modified with a proprietary selection of supplements and growth factors that provide greater lot-to-lot consistency. However, we find here that it cannot sufficiently sustain dividing Caco-2 cells and is not a viable long-term solution. Second, we describe a new variation of 3-day Caco-2 epithelia, which we have deemed thrifty, rapid intestinal monolayers (TRIM). TRIM offer fast differentiation and more palatable costs compared to other systems of fully-formed Caco-2 monolayers. Here, we describe the process of their formation and compare their gene expression and permeability behavior to two other common Caco-2 models: 21-day monolayers and the Corning HTS 3-day system.

MATERIALS AND METHODS

Materials

Penicillin/streptomycin, trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA), phosphate buffer saline (PBS), rat tail Collagen I, calcein, DAPI, Hoechst 33342, Alexa Fluor® 488 Phalloidin, Alexa Fluor® 488 anti-Claudin-1 antibodies, Alexa Fluor® 594 Anti-ZO-1 antibodies, and ClearMount™ solution were purchased from Life Technologies® (Thermo Fisher subsidiary, Carlsbad, CA, USA). The cDNA reverse transcriptase kit (Applied Biosystems) and primers for GAPDH (Hs02758991_g1), ZO-1 (Hs01551861_m1), and Claudin-1 (Hs00221623_m1) were also ordered from Life Technologies using the best coverage primer/probe set. Caco-2 cells were purchased from American Type Culture Collection® (ATCC, Manassas, VA, USA). Dulbecco's Modified Eagles Medium (DMEM), fetal bovine serum (FBS), Seradigm FB Essence (FBE), Falcon® 225 cm^2 tissue

culture flasks, Corning® 1.0 μm porous support Transwell® plates and BioCoat™ HTS plate kits, Falcon® 24-well plates, sodium butyrate, Corning® MITO+ serum extender, and Rhodamine 123 (Rhod123) were obtained from VWR® (Radnor, PA, USA). 4 kDa FITC-labelled dextran (FITC-DX4), p-Nitrophenyl phosphate (p-NPP), p-nitrophenol (p-NP), 1-phenylpiperazine (PPZ), low molecular weight chitosan, and sodium lauryl sulfate (SLS) were purchased from Sigma-Aldrich® (St. Louis, MO, USA).

Caco-2 Cell Culture

Caco-2 lines were screened to ensure mycoplasma-free conditions by direct DNA staining with Hoechst 33342 (26). Cells were cultured in DMEM supplemented with 10% FBS, 100 IU/ml of penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml Amphotericin B ("Caco-2 media"). Cultures were incubated at 37°C in 5% CO_2 and 100% relative humidity. The cells were subcultured with 0.25% trypsin-EDTA and subsequent passaging every 3 to 4 days at ratios between 1:3 and 1:8. Cells at passage numbers 20–60 were used for experiments.

Cell Proliferation Experiments

Caco-2 lines at p30 were transferred into DMEM supplemented with either FBS (10% *v*/v) or FBE (5, 10, 15, or 20% v/v). All media also contained 100 IU/ml of penicillin, 0.1 mg/ml streptomycin, and 0.25 μg/ml Amphotericin B. Each group of cells was maintained for several more passages in its respective media composition. At passage numbers 32, 37, and 40, the cells were seeded into 24-well plates at 1.5×10^5 cells/well in their respective media compositions. Every day for four days, three wells of each cell population were rinsed to remove dead cells and debris, trypsinized, and counted using a hemacytometer.

Collagen Coating for Transwell Plates

Transwell inserts were coated with collagen I from rat tails per the supplier-provided thin coating procedure. Briefly, collagen (5 μg per cm^2 of transwell membrane surface) was diluted into 20 mM acetic acid solution. The mixture was added to the apical compartments of the wells and incubated at room temperature for one hour. Plates were then rinsed three times with PBS and either used immediately or dried under sterile conditions and stored at 4°C for up to three months.

Corning® HTS Monolayers

Cells were suspended in basal seeding medium (BSM) provided in the HTS kit, seeded at a density of 2×10^5 cells per well in the supplied HTS plate, and incubated for 24–48 h. The media was then changed to the enterocyte differentiation medium (EDM) and incubated for 48 h. The trans-epithelial electrical resistance (TEER) was monitored to confirm proper barrier formation, and only monolayers with TEER values in the acceptable range (14) of 150–750 Ω·cm^2 were utilized for further experiments.

TRIM Monolayers

Cells were suspended in DMEM supplemented with MITO+ serum extender according to the manufacturer's instructions. They were seeded at a density of 2×10^5 cells per well on collagen-coated transwell supports and incubated for 24–48 h. The media was then changed to DMEM supplemented with the growth factor blend MITO+ and 2 mM sodium butyrate, a known component of EDM from the HTS kits, and incubated for 48 h. The TEER was monitored and only monolayers with TEER values of 150–750 Ω·cm2 were used for further experiments.

21-Day Monolayers

Cells were suspended in DMEM supplemented with 10% FBS ("21-day media") and seeded at a density of 2×10^5 cells per well on collagen-coated transwell supports. The media was then aspirated and replaced every 2–3 days for 21 days. TEER was monitored to confirm proper barrier formation, and only monolayers with TEER values of 150–750 Ω·cm^2 were used for further experiments.

Monolayer Permeability and Permeation Enhancer Experiments

Caco-2 monolayer trays were transferred to 24-well plates containing 1 ml DMEM per well and were allowed to equilibrate for 30 min before recording TEER values using a Millicell® voltohmmeter. Fluorescent paracellular diffusion markers were applied at 0.5 mM (calcein), 0.2 mM (FITC-DX4), or 0.1 mM (Rhod123) into the apical side of the monolayers. After one hour, media in the basal chambers was sampled and its fluorescence was measured at 495/515 nm (calcein), 485/515 nm (FITC-DX4), or 510/535 nm (Rhod123) using a Biotek Synergy2 plate reader. Application of calibration curves yielded the amount of mass transferred across each monolayer, which was used in the permeability equation $P_{app} = \frac{\Delta M}{C_a A \Delta t}$, where P_{app} is the apparent permeability through the monolayer, ΔM is the marker mass accumulated in the basal compartment, C_a is the apical marker concentration, A is the monolayer area, and Δt is the time between samples.

To characterize the effects of permeation enhancers, chitosan (1 mg/ml), SLS (0.1 mg/ml), or PPZ (1 mg/ml) was dissolved in EDM and applied to the apical chambers with the fluorescent markers. Negative control wells received fresh EDM. DMEM in the basal compartments was refreshed once per hour to maintain sink conditions for diffusion. TEER and permeability measurements are expressed as the ratio of each monolayer's permeability for the first hour after permeation enhancer addition to its permeability before treatment, normalized to any change in untreated control monolayers during that time.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase activity was measured as previously described (27). Briefly, monolayers or mouse intestinal segments were washed with calcium- and magnesium-supplemented PBS. A reaction buffer containing p-nitrophenyl phosphate was added, and the samples were incubated at room temperature. Ten minutes later, 100 µL buffer samples from each well were transferred to a 96-well plate, with 50 µL/well of 0.5 M NaOH, and the temperature was decreased to 4°C to halt the reaction. The plate reader was used to measure the absorbance of the samples at 405 nm, which was compared to a calibration curve of p-nitrophenol to determine the amount of p-nitrophenyl phosphate degraded by alkaline phosphatase. For monolayers, the amount of p-nitrophenol produced was normalized to the area of the porous membrane filter. For intestinal samples, the size of the section and a surface amplification factor for the villi (28) were used to determine epithelial area for normalization.

Gene Expression by qPCR

RNA was isolated from Caco-2 cells, monolayers, or epithelial cells scraped off of mouse intestinal segments, using Qiagen RNeasy Kit according to the manufacturer's protocol. Reverse transcriptase PCR was carried out using the high capacity cDNA reverse transcription kit according to the manufacturer's protocol.

Quantitative PCR (qPCR) was performed using the ViiA 7 Real-Time PCR system and Taqman universal PCR master mix (Applied Biosystems). Each qPCR reaction contained a total reaction volume of 20 µl (100 ng cDNA +10 µl Taqman mastermix +1 µl Tagman endogenous control +1 µl Taqman gene expression). All runs were performed in comparative Ct mode with a temperature profile of 50°C for 2 min, 95°C for 10 min, and 40 cycles of [95°C for 15 s and 60°C for 1 min]. All qPCR samples were tested with three biological replicates and three technical replicates each. The expression of each tight junction mRNA was normalized to expression of the housekeeping gene GAPDH and is displayed relative to expression levels for undifferentiated Caco-2 cells.

Confocal Microscopy

Monolayers or excised intestines were rinsed with PBS and fixed in 4% formaldehyde. They were permeabilized with 0.2% Triton-X100 and blocked with 0.2% BSA solution to limit non-specific antibody binding, then incubated for one hour with staining solutions. The staining solutions contained Hoescht 33,342 (20 nM, 350 nm/461 nm) to mark nucleic acids, AlexaFluor 488® Phalloidin (5 units/ml, 495 nm/518 nm) to label actin, AlexaFluor 488® Anti-Claudin 1 antibodies (50 µg/ml, 495 nm/518 nm), and/or AlexaFluor® 594 Anti-ZO-1 antibodies (50 µg/ml, 590 nm/617 nm) in PBS with 0.2% BSA. After staining, the monolayers and membranes were mounted on slides using ClearMount™ solution and sealed under coverslips using clear nail polish, while intestinal segments were sectioned, suspended in PBS, and mounted to slides using rubber spacers and cover slips.

Prepared slides were imaged at 63x magnification using a Zeiss LSM 700 confocal microscope with ZEN 2012 SP1 software. Images were captured using a Plan-Apochromat 63x/1.40 Oil DIC objective and an X-Cite Series 120Q laser source exposing at 405, 488, and 555 nm. ImageJ (NIH) image processing software was used to prepare confocal images for publication. Upper and lower thresholds were narrowed slightly to remove background noise and improve visibility of the signals. All images were processed with the same thresholds and display lookup tables, which were linear throughout their ranges. Images were converted from their original 16-bit format to RGB color for saving and arrangement into figures. No other manipulations were performed.

Mouse Studies

All mouse experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at Carnegie Mellon University (Pittsburgh, PA, USA) under protocol number PROTO201600017 and performed in accordance with all institutional, local, and federal regulations. C57BL/6 mice were either purchased from Charles River Laboratories (Wilmington, MA, USA) or obtained from an institutionally managed breeding colony. Prior to experiments, mice were housed in cages of no more than five animals, with controlled temperature (25°C), 12 h light-dark cycles, and free access to food and water. Mice used in this study were male and 12–21 weeks old. Mice were fasted 12 h the night before an experiment to limit the variability caused by food matter and feces in the gastrointestinal tract.

Intestinal Permeability to Dextran

For dextran permeability studies, fasted mice were anesthetized and their intestines surgically exposed. They received direct injections (2 µL/g volume) of permeation enhancers (200 mg/kg dose of SLS, 32.4 mg/kg PPZ, PBS control)

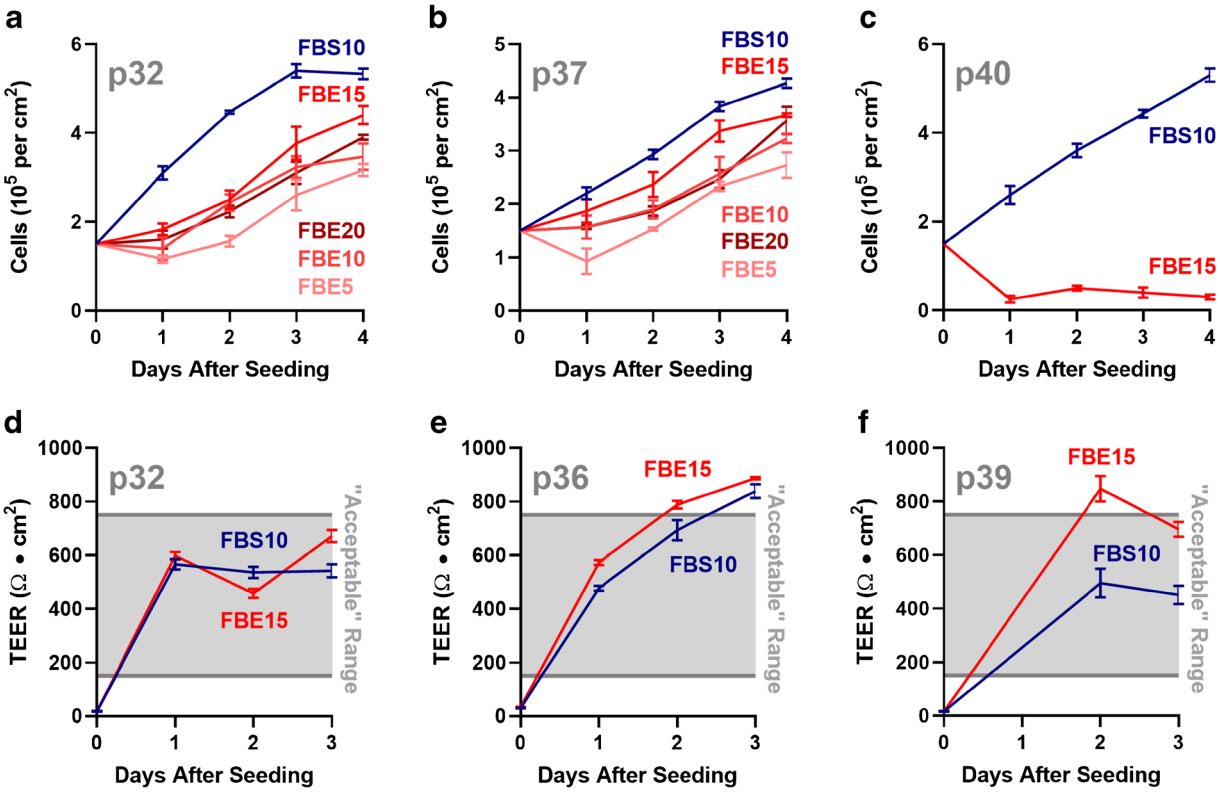

Fig. 2 Lower-cost FBS alternative FB Essence (FBE) is of limited use in maintaining Caco-2 cells for intestinal monolayer production. All graphs compare Caco-2 cells originating from the same population (i.e. growing in the same flask) until the split into different bottles containing different FBS and FBE content media at passage 30 (p30). (**a**) At two passages (**b**) and seven passages after transfer into media containing varying amounts of FBE, cells growing in 15% FBE (FBE15) proliferate at almost the same rate as cells growing in the traditional 10% FBS (FBS10). (**c**) However, by ten passages in the 15% FBE media, the cells cease to grow and divide. (**d**) At two passages, (**e**) six passages, and (**f**) nine passages after transfer, Caco-2 cells growing in 15% FBE media formed monolayers with comparable transepithelial electrical resistance (TEER) to cells grown in 10% FBS. Gray windows represent the range of acceptable TEER values for experimentation. Error bars display s.e.m. ($n = 3$ for cell proliferation, $n = 12$ for TEER)

and FITC-DX4 (10 mg/kg) directly into the duodenum. At 0.5 h post-injection, blood was collected and centrifuged. The FITC concentration in the serum was measured by reading for fluorescence on the plate reader (485/515 nm) and comparing to a unique calibration curve for each experiment.

Statistics

All data are presented as the arithmetic mean of the given "n" number of biological replicates (individual animals or number of in vitro cell culture wells), and error bars display the standard error of the mean. For statistical significance, two-tailed Student's t-tests were used to calculate p values, and $p < 0.05$ was considered to indicate statistical significance.

RESULTS AND DISCUSSION

FB Essence for Reduced Cell Line Maintenance Costs

We began by asking whether FB Essence (FBE) could replace fetal bovine serum (FBS) in the maintenance of the Caco-2 cell line. To do this, we first optimized FBE content for Caco-2 growth and proliferation. Caco-2 cells at passage number 30, previously growing in standard 10% FBS-supplemented media, were split into five groups: one continued in 10% FBS media, and the others were transferred into flasks with 5%, 10%, 15%, or 20% FBE. At pre-determined passage numbers, cells were examined for their proliferative ability. After two passages (Fig. 2a) and seven passages (Fig. 2b), the cells growing in 15% FBE media outperformed each of the other FBE groups. However, their growth still lagged behind the cells in the FBS control media. To this end, the FBE cell flasks required higher passage ratios to maintain the same schedule as the FBS controls (e.g. a 1:3 split instead of 1:4 or 1:6 to cover the same three day span). Surprisingly, at between seven and nine passages in the FBE media, most of the cells stopped dividing altogether. Only the 15% FBE group survived to ten passages, but even those cells lost the ability to adhere and proliferate (Fig. 2c). This is consistent with the eventual decline in cell growth seen in studies of other FBS alternatives for Caco-2 proliferation (24). Thus, FBE can be used to supplement media for short-term Caco-2 maintenance, but it should not be used for long-term propagation of the cell line.

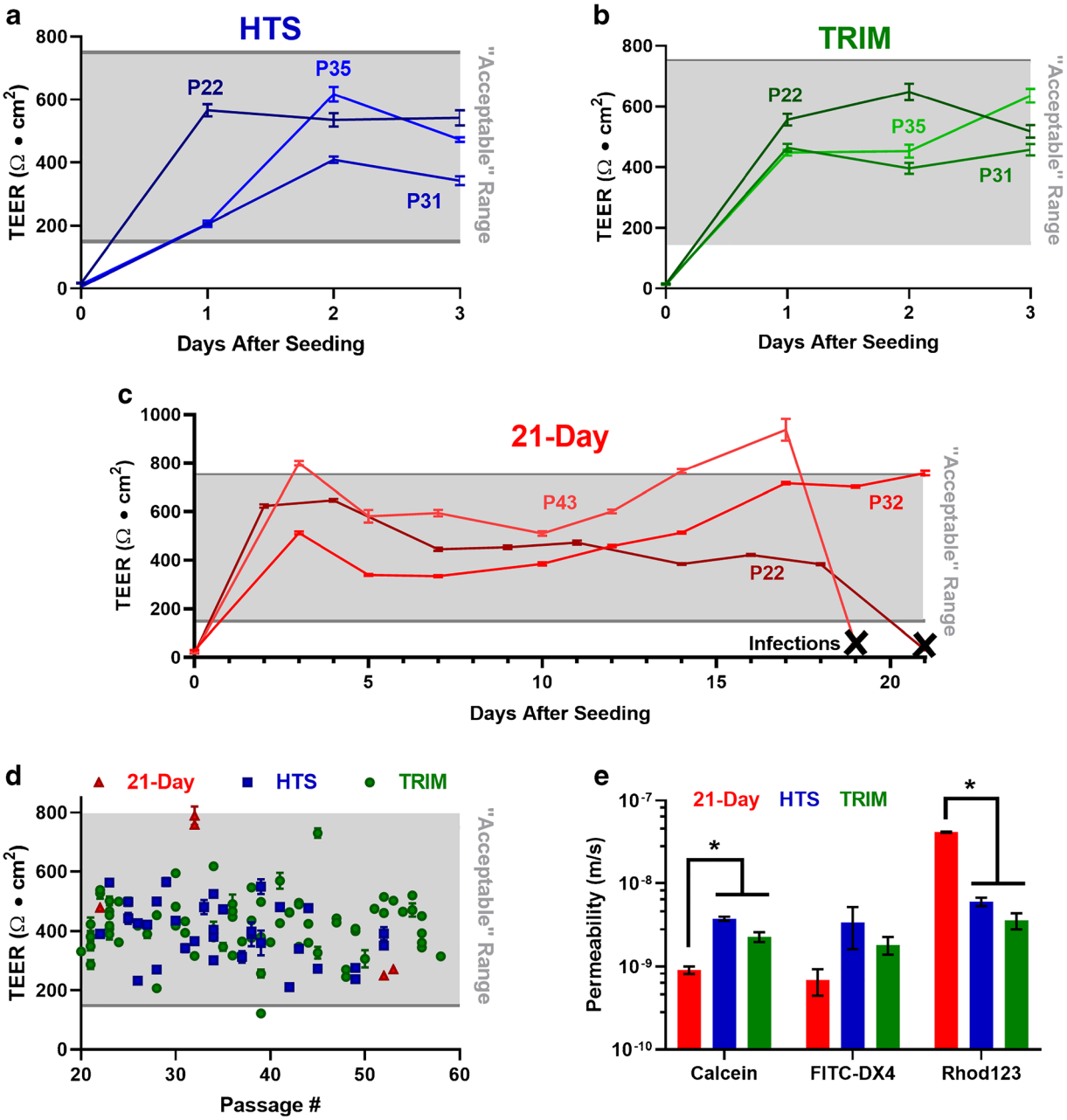

Fig. 3 The three monolayer preparation methods generated similar TEER values but some differences in permeability. (**a**) In the HTS system, passage number causes early discrepancies in barrier quality, but these differences diminish by the third day. (**b**) TRIM monolayers were not affected by passage number. (**c**) 21-day monolayers attain high barrier function by the third day, regardless of passage number, and maintain high TEER as the cells continue to differentiate. However, high rates of infection among 21-day plates leads to failure of many monolayers. (**d**) The vast majority of all monolayers from each of the three systems had TEER values in the "accepted range". Each point represents an entire 24-well plate of monolayers. (**e**) Monolayers from the 21-day system exhibited lower paracellular permeability to calcein and 4 kDa FITC-Dextran (FITC-DX4) and higher transcellular permeability to Rhodamine 123 than the 3-day systems. Error bars display s.e.m. (n = 12-24 for TEER, n = 3 for permeability). * $p < 0.05$ by two-tailed t-test

Because 15% FBE was adequate for short-term Caco-2 maintenance, we hypothesized that cells cultured with this serum composition would also be able to differentiate into monolayers, at least for as long as their ability to proliferate persisted. To test this, we examined the ability of the cell populations growing in 15% FBE and 10% FBS (control) to form monolayers with the 3-day HTS Transwell® system from Corning®. After two (Fig. 2d) and six (Fig. 2e) passages, there was little difference in transepithelial electrical resistance (TEER) values between the two populations, suggesting that similar monolayers had developed. TEER measures the ability of ions to pass through the monolayers and has been shown

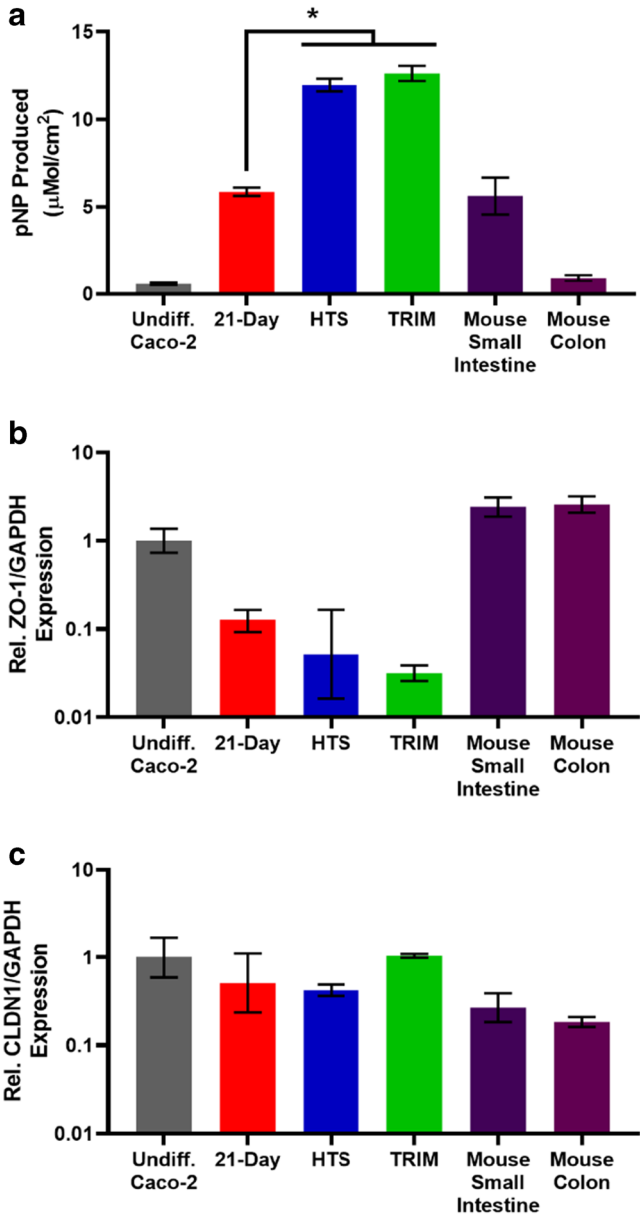

Fig. 4 Gene expression varies between different monolayer systems and between Caco-2 and intestinal tissue. (**a**) Activity of alkaline phosphatase, a common differentiation marker for intestinal enterocytes, is significantly higher in 3-day monolayer systems than in 21-day monolayers or mouse intestinal mucosa. (**b**) In all cases, Caco-2 monolayers exhibited less expression of tight junction protein ZO-1 than intestinal cells taken from mouse intestines. (**c**) While claudin-1 (CLDN1) expression was similar between 3-day monolayer systems and mouse small intestines, 21-day monolayers exhibited higher claudin-1 levels. Error bars display s.e.m. (n = 3). * $p < 0.05$ by two-tailed t-test

to correlate inversely with permeability through the tight junctions (29,30). Thus, higher TEER relates to more complete formation of tight junctions within the monolayers (31,32). Interestingly, even as their proliferation slowed, the cells cultured in 15% FBE media formed proper monolayers (Fig. 2f). Most likely, this is due to the high seeding density of the HTS system, allowing the cells to achieve confluence on the transwell membrane without needing to divide.

Based on their comparable TEER behavior to current Caco-2 models, cells from 15% FBE media could likely be used as a short-term measure for cost saving. However, the decline of cell populations after around 10 passages means that any lab using them would need a continuously maintained, separate line of Caco-2 in 10% FBS or enough cells stored to thaw new populations of cells each month. On top of this operational complication, the only successful media formulation using FBE required a higher concentration (15%) than the formulation using FBS (10%). As of June 2019, the cost of FBE is approximately half that of FBS ($160 vs $340 per 500 ml). For most labs, the resulting 30% reduction in serum costs would likely not outweigh the cost increases associated with the shortened life of the cell line or the possibility that FBE alters long-term gene expression in the cell line.

TRIM as an Alternative to HTS and 21-Day Monolayers

As a second strategy for reducing the time and cost of in vitro screening with Caco-2 cells, we developed a system termed thrifty, rapid intestinal monolayers (TRIM). TRIM combine the abbreviated, 3–4 day formation time of the HTS system with the less expensive materials of 21-day monolayers. As with any new system, we set out to determine how its behavior compared to both established cell culture models and the tissues they are meant to represent. To start, we examined the development of TEER over the course of TRIM formation compared to the same cells differentiating into two established Caco-2 models: Corning® HTS, and 21-day monolayers. TRIM (Fig. 3a) and HTS (Fig. 3b) demonstrated similar behavior, with TEER reaching the acceptable range within 24 h and persisting for three days, regardless of cell passage number. Similarly, 21-day monolayers reached high TEER values within 2–3 days after seeding (Fig. 3c). Values remained steady during the designated junction maturation period, provided the cells did not become infected. For each of the systems, TEER was within the acceptable resistance range on the pre-determined experiment day in almost all iterations of monolayer development (Fig. 3d).

In addition to TEER development, apical-to-basal permeability of diffusion marker molecules is a critical experimental characteristic that we wanted to understand in the context of the three systems. First, we examined the permeation of two hydrophilic molecules through the tight junctions: calcein (~620 Da) and 4 kDa, FITC-labelled dextran (FITC-DX4). In both cases, paracellular transport was comparable across all three monolayer systems, registering within the same order of magnitude (Fig. 3e), though the tight junctions developed in the 21-day system were slightly better at excluding both

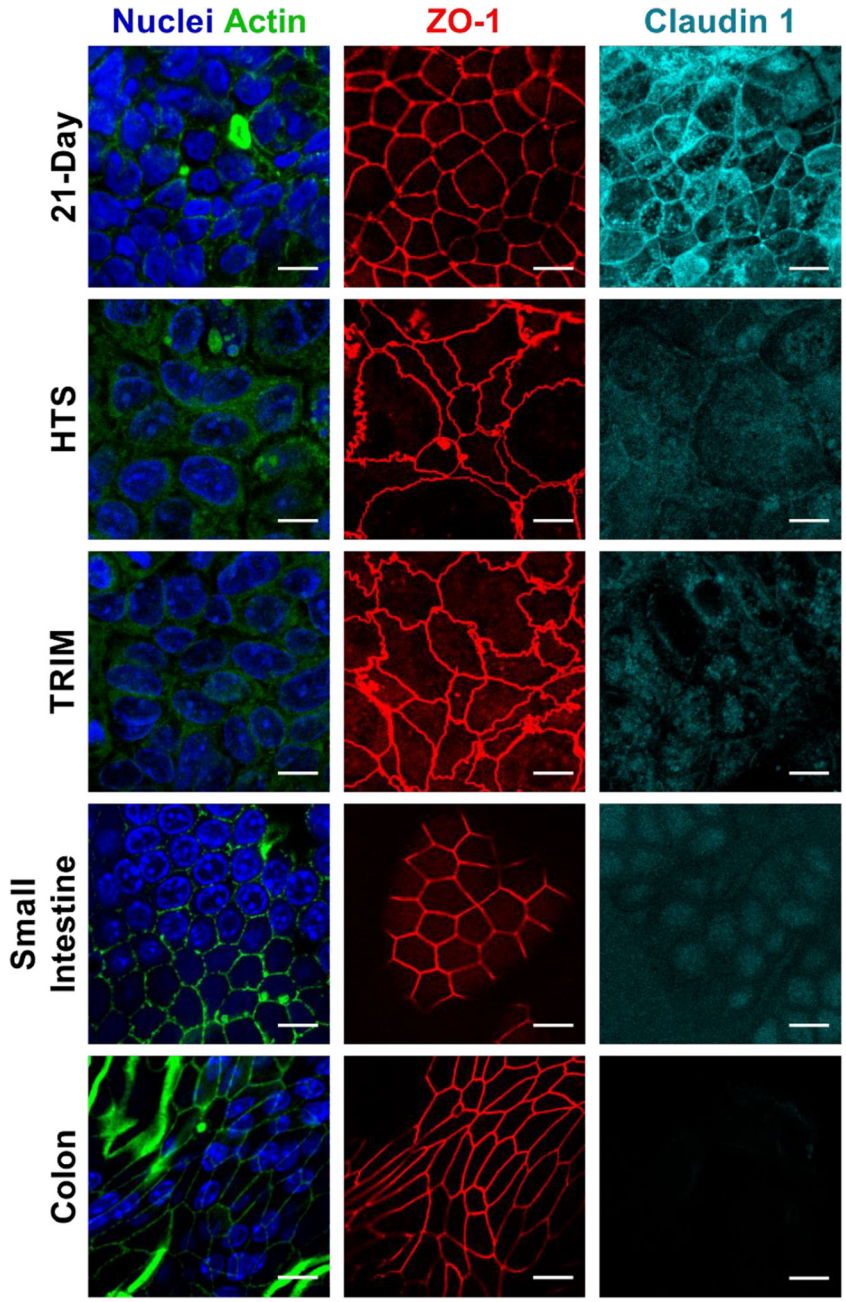

Fig. 5 3-day Caco-2 systems develop similar tight junctions to one another but differ slightly from the 21-day system. Among the three types of monolayers, the shape and arrangement of nuclei and actin displayed no notable differences. However, the tight junction protein ZO-1 forms predominantly straight, smooth structures in the 21-day system, while both 3-day systems display ruffling in the ZO-1 pattern. Another tight junction protein, Claudin 1 is much more clearly expressed in the 21-day system than in the 3-day systems. In HTS and TRIM monolayers, as well as both intestinal segments, the Claudin 1 signal is so faint that it is barely indistinguishable from background fluorescence. Scale bars = 10 μm

molecules. To assess transcellular permeability, we next applied the fluorescent marker rhodamine 123 (Rhod123) to the apical side of monolayers. Rhod123 is sufficiently hydrophobic to diffuse through the cell membrane and is a known P-glycoprotein substrate (22). Thus, increased Rhod123 transport in the apical to basal direction implies that less P-glycoprotein efflux activity is pumping the marker from the cytosol back into the apical compartment. Permeability of Rhod123 was substantially higher in 21-day monolayers than in HTS or TRIM (Fig. 3e). This has been previously reported with other abbreviated monolayer systems (22) and implies that such monolayers have greater P-glycoprotein activity.

Gene Expression in Monolayer Models

The observed differences in P-glycoprotein activity prompted us to question how TRIM development affects gene expression of various proteins when compared to other monolayer models, as well as actual mouse intestines. To begin, we examined the activity of alkaline phosphatase, a brush border enzyme used as a differentiation marker for Caco-2 cells (1,27,33), by measuring conversion of p-nitrophenyl phosphate to p-nitrophenol (Fig. 4a). TRIM exhibited approximately the same enzyme activity as the HTS monolayers, and both were significantly higher than 21-day monolayers

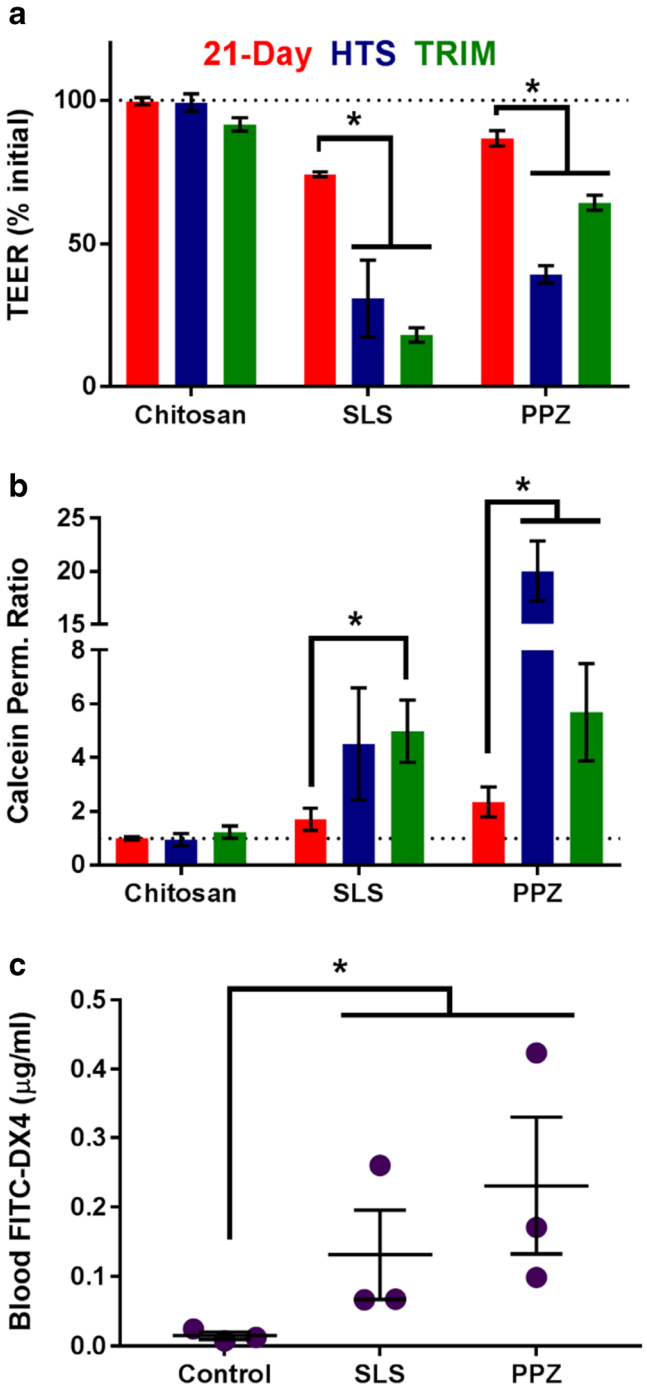

Fig. 6 Caco-2 response to chemical permeation enhancers is dependent on the monolayer system used. (**a**) By trans-epithelial electrical resistance (TEER), the 3-day monolayer systems indicated greater permeation enhancing power of species tested than did the 21-day monolayers. (**b**) The standard relationship of lower TEER corresponding with higher mass permeability held up across all three monolayer systems. Calcein perm. Ratio indicates the fold change in each monolayer's permeability following treatment, normalized to behavior of untreated control wells. (**c**) As predicted by the 3-day monolayer systems, SLS and PPZ enhanced accumulation of intestinally injected FITC-DX4 in the bloodstream of mice. Error bars display s.e.m. (n = 3 for cells, n = 3 for mice). * $p < 0.05$

Table I Cost and Time Comparisons Among 24-well, Caco-2 Monolayer Systems. Costs are Based on Prices as of June 2019. Calculations do not Include Cost or Time Associated with Ongiong Maintenance of the Cell Line

	21-Day	HTS	TRIM
Transwell Plate	109.21		109.21
Collagen	0.14		0.14
Acetic Acid	0.01		0.01
HTS PlateKit		366.11	
DMEM	4.32	1.30	3.12
FBS	59.76		
Sodium Butyrate			0.08
MITO+			4.26
Cost Total (USD)	173.53	367.41	116.81
Collagen Coating	12		12
Preparing Media	10	5	10
Seeding Cells	40	40	40
Media Changes	160	15	15
Time Total (Min)	222	60	77
Time Total (Hours)	3.7	1	1.3

and mouse small intestines. Interestingly, colon samples expressed the lowest level of enzyme activity. However, it should be noted that data for mouse intestinal samples rely on application of published conversion factors for epithelial surface area (28), which introduces some uncertainty as to the absolute values.

Two other proteins of interest in the intestinal epithelium are the tight junction proteins ZO-1, which anchors junctions to the cytoskeleton, and Claudin 1, which partially spans the intercellular junction space (31). Examining cell lysates by qPCR demonstrates that ZO-1 gene expression is consistent between TRIM, HTS, and 21-day monolayers (Fig. 4b), though it is far lower in all three systems than in mouse colon or intestines. In contrast, expression of Claudin 1 is comparable between TRIM, HTS, and small intestines (Fig. 4c), while 21-day monolayers may slightly overexpress the gene. Taken together, these differences in protein activity and gene expression show that no single monolayer system provides a completely accurate representation of in vivo intestines, and care should be taken in choosing the appropriate assay for a given application.

Imaging Analysis of Monolayer Tight Junctions

Having observed significant differences in the gene expression of tight junction proteins among the five models, we were curious as to how TRIM would compare to the other models in the spatial arrangement of the junction proteins. We used confocal microscopy to visualize nuclei, actin, ZO-1, and Claudin 1 (Fig. 5).

Phalloidin and DAPI staining revealed few differences in morphology across the five samples, though the nuclei in the 21-day system were smaller and more irregular than those in the other models. In contrast, the ZO-1 encircling the cells at the tight junctions was noticeably variable. In the 21-day monolayers and excised intestines, the ZO-1 smoothly and regularly ringed the cells, while in both TRIM and HTS monolayers, the ZO-1 displayed ruffling at the edges of the cells. This change in morphology has previously been seen in Caco-2 cells treated with nanostructures to open the tight junctions (34), suggesting a connection between the ruffling observed in the images of TRIM and HTS monolayers and the slightly higher tight junction permeability to calcein and FITC-DX4 observed in this study (Fig. 3e). Finally, as we predicted based on the gene expression experiments in the previous section, the signal for Claudin 1 was strongest in 21-day monolayers and weakest in the colon, with TRIM showing weak but observable staining around the cell perimeters (Fig. 5). These differences in protein localization and morphology again indicate that TRIM and other monolayer systems each have particular strengths and weaknesses with respect to representing the intestinal epithelium accurately.

Utility in Permeation Enhancer Screening

Given the observed differences between tight junction expression and morphology, we ultimately asked how the models would differ in their predictions of permeation enhancer efficacy. The monolayers were treated with three permeation enhancers, each known to increase paracellular permeability (29,35–38): chitosan, 1-phenylpiperazine (PPZ), and sodium lauryl sulfate (SLS). As measured by TEER, 21-day monolayers were less affected by these concentrations of paracellular permeation enhancers than TRIM or HTS monolayers (Fig. 6a), likely due in part to differences in how tight junction proteins are expressed in each system. This trend was confirmed by increased passage of the diffusion marker calcein through treated TRIM and HTS monolayers (Fig. 6b). The 21-day monolayers did not indicate a significant increase in transport for any of the three permeation enhancers.

When evaluated in vivo via direct injection into the intestines of mice, the SLS and PPZ permeation enhancers significantly increased the transport of the macromolecule FITC-DX4 out of the intestines and into the bloodstream, as predicted by the behavior of the TRIM and HTS systems. As a result, enhancer-treated mice displayed significantly accumulation of FITC-DX4 in the bloodstream (Fig. 6c). This indicates that these systems are particularly useful for screening potential intestinal permeation enhancers before advancing to animal models (39).

Relative Costs of Monolayer Development

Because each of the three monolayer systems examined here have distinct strengths and weaknesses, we anticipate that cost and time input will be an important factor for experimental design in many studies. Based on supply costs as of June 2019, TRIM are the least expensive monolayers to produce, and cost $117 for a 24-well plate, or approximately $4.88 per well. The 21-day had 50% higher costs, costing $174 per plate and $7.25 per well. Finally, HTS expenses are 200% higher, costing $368 per plate and $15.33 per well (Table I). Even for just one researcher operating at a rate of one experiment per week, this adds up to over $800 per month savings using TRIM over HTS. There is a slight trade off in time input, as TRIM require an extra 12 min per plate for collagen coating (Table I), when performed in batches of 5. However, TRIM clock in at just over one hour per plate, while 21-day monolayers require nearly four hours over three weeks. On top of this, 21-day plates are highly prone to infection, as we have shown here (Fig. 3c). The high failure rate further increases the time input to 5–8 h per successful monolayer plate, greatly exceeding the approximate one hour for HTS and TRIM. Finally, TRIM and HTS methods drastically reduce the lag time between experiments, allowing for better planning and more efficient work flow.

CONCLUSION

The development of faster, less expensive Caco-2 epithelial models is needed to accelerate research on gastrointestinal disease and oral drug delivery. Like many other serum alternatives, FBEssence does not provide a long-term, cost-saving strategy for Caco-2 culture. However, TRIM and HTS monolayer systems offer experimentally comparable and less labor-intensive alternatives to 21-day monolayers, especially for research examining intestinal permeability. Furthermore, TRIM can be constructed for less than one third of the cost of HTS monolayers. For the particular characteristics studied here, each system had its own strengths and weaknesses as models of in vivo intestinal tissue. To this end, Caco-2 monolayers will not always accurately predict the extent of permeability change or gene expression in intestinal tissues. Like all cell culture models, their use must be validated to accurately reflect transport and other behavior for each researcher's compound

of interest. However, they remain critical screening tools prior to in vivo experimentation and can substantially reduce the number of animals required (39). In our opinion, TRIM are a valuable addition to Caco-2 intestinal monolayer options, allowing researchers to trim expenses and time inputs for their research.

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