Evaluating the technical quality of a triculture gut model to test particle permeability

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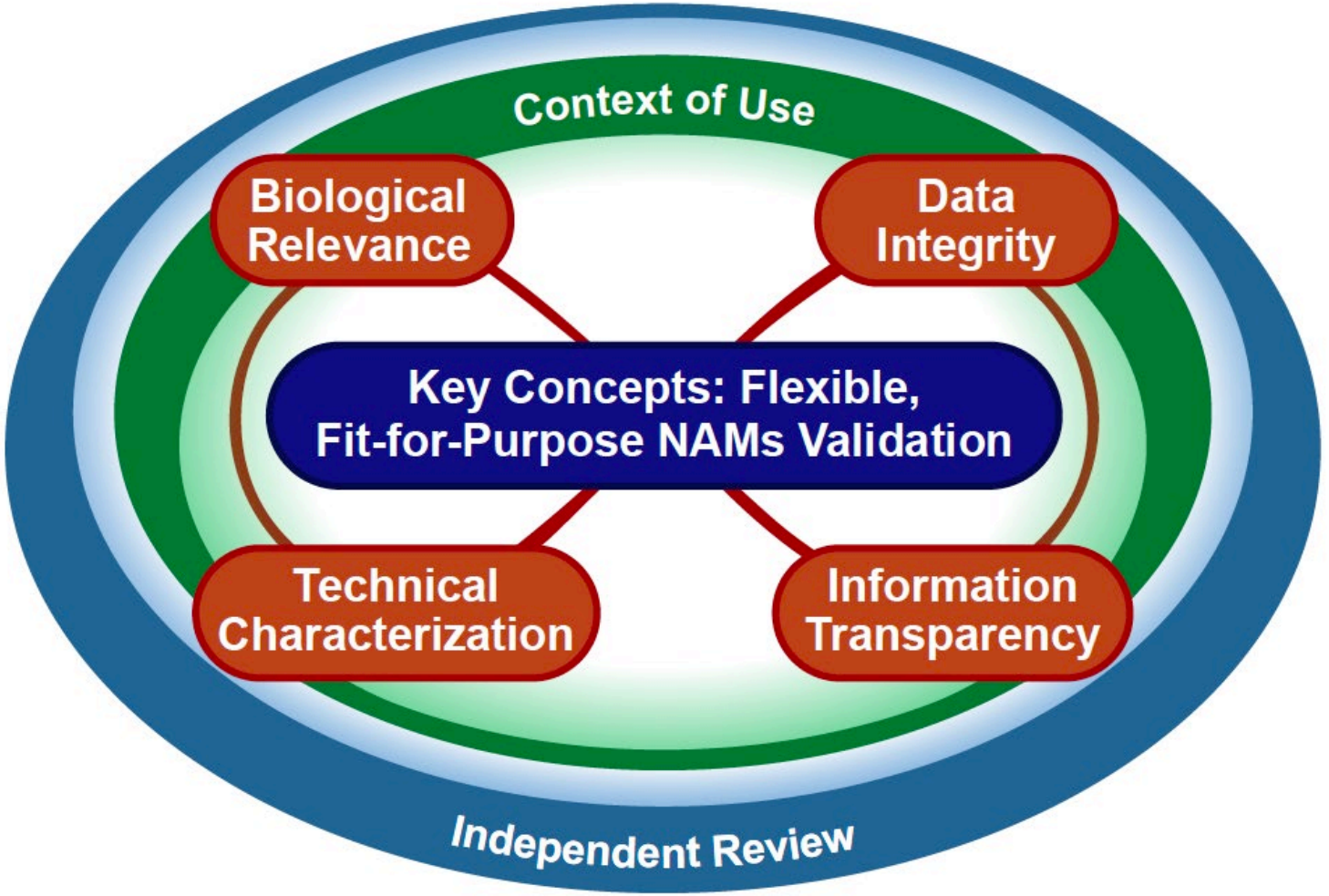
National Institute of Standards and Technology
U.S. Department of Commerce

Key Concepts to Consider During Development and Implementation of Flexible, Fit-for-Purpose NAMs Validation Strategies



Final Draft ICCVAM Validation Report, Figure 1
(adapted from van der Zalm et al. 2022 Arch Tox)

https://ntp.niehs.nih.gov/go/ICCVAM-submit

Technical Framework Manuscript

ALTEX, accepted manuscript
published July 15, 2022
doi:10.14573/altex.2205081

Bench Marks!

Technical Framework for Enabling High-Quality Measurements in New Approach Methodologies (NAMs)

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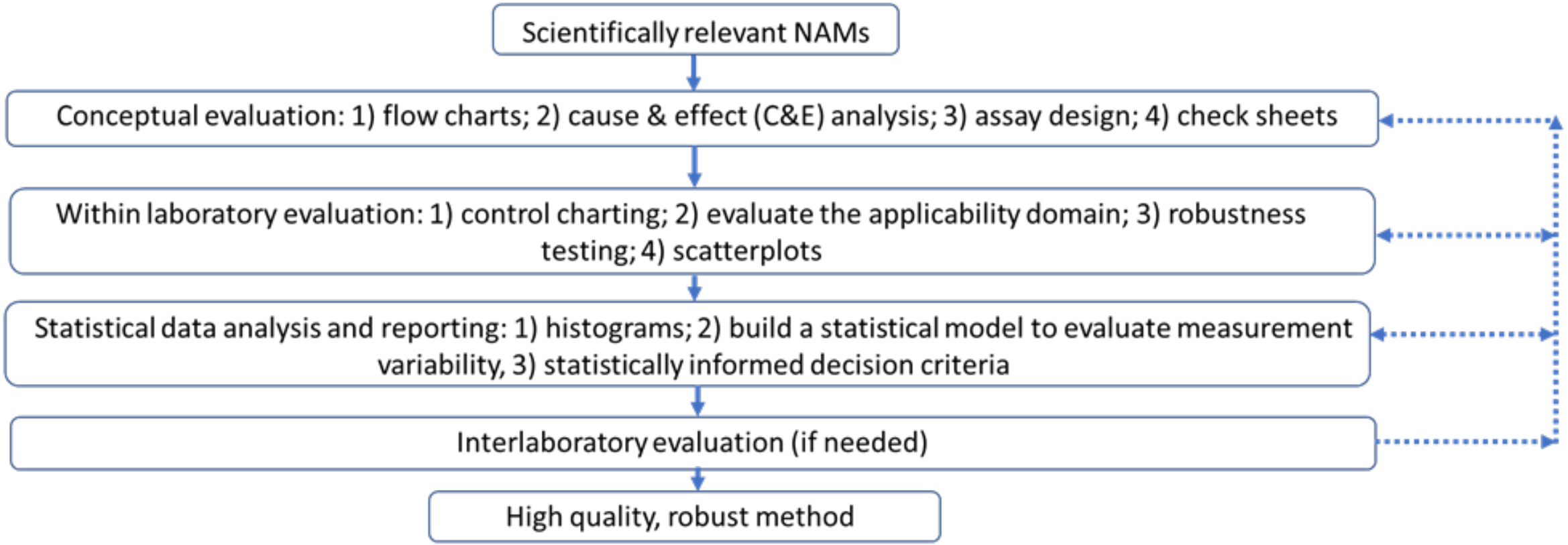
Technical Framework for High Quality NAMs

Collaborative project with CPSC, NICEATM, DOD, EMPA, NIST

- To yield reproducible NAM results across time and among laboratories, the framework includes a series of inter-related steps that describe
  - How to apply basic quality tools (cause-and-effect analysis, flow charts, control charts, etc) to improve confidence in NAMs
  - Approaches for adding statistical confidence to decisions based on NAM results
  - There may be tradeoffs though with more controls potentially leading to higher costs

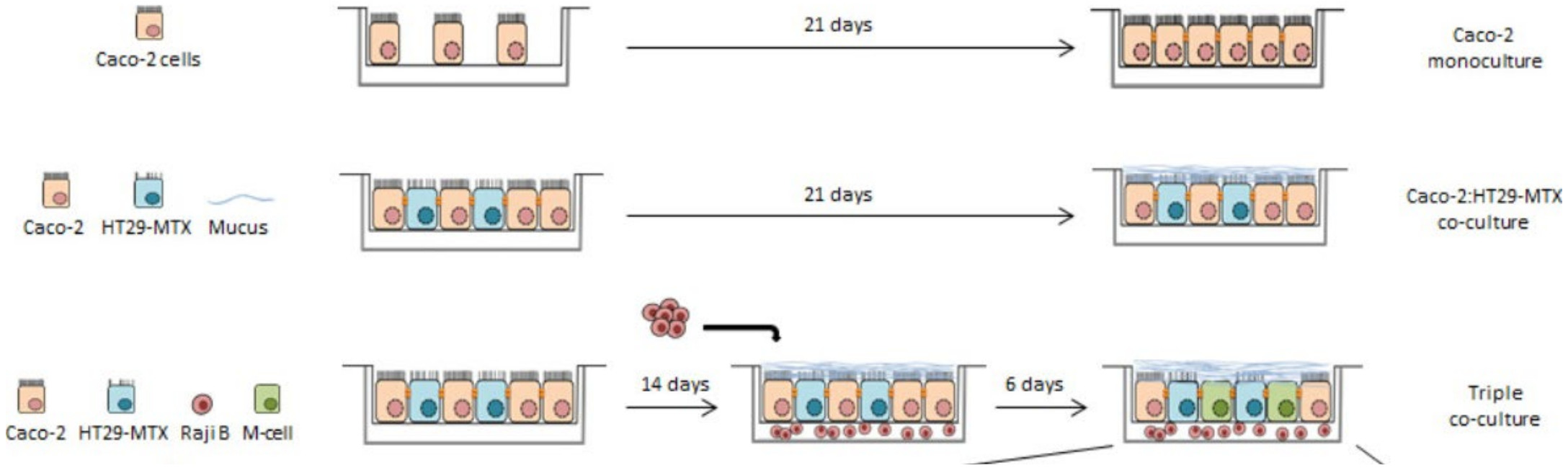
Petersen, E. J., Elliott, J. T., Gordon, J., Kleinstreuer, N., Reinke, E, Roesslein, M., Toman, B. 2023, Altex. https://doi.org/10.14573/altex.2205081

Technical framework for high quality NAMs



Petersen, E. J., Elliott, J. T., Gordon, J., Kleinstreuer, N., Reinke, E, Roesslein, M., Toman, B. 2023, Altex. https://doi.org/10.14573/altex.2205081

Three different *in vitro* cell models



Araujo et al. 2013

Cell seeding:
Caco 2/HT-29 total cells apical side: 250,000 cells per well in a 90:10 ratio
Raji B* total cells basolateral side: 500,000 cells per well change every 2-3 days and added at day 14

Goals of this model: More complexity and improved physiological relevance

Key parameters and control measurements for the triculture gut model

Cell viability and metabolic activity: MTS assay

Mucus production and distribution: cell staining + microscopy, ELISA

Barrier integrity during 3D tissue formation: transepithelial electrical resistance (TEER)

Permeability of reference compounds: molecular and particle controls (e.g., Lucifer yellow, FITC-dextran, fluorescent particles)

Proportion of M cells: electron microscopy (?)

Varying results with Alcian blue staining in the literature

Alcian blue is a basic dye with affinity to acidic mucin glycoproteins.

Araújo et al. 2013

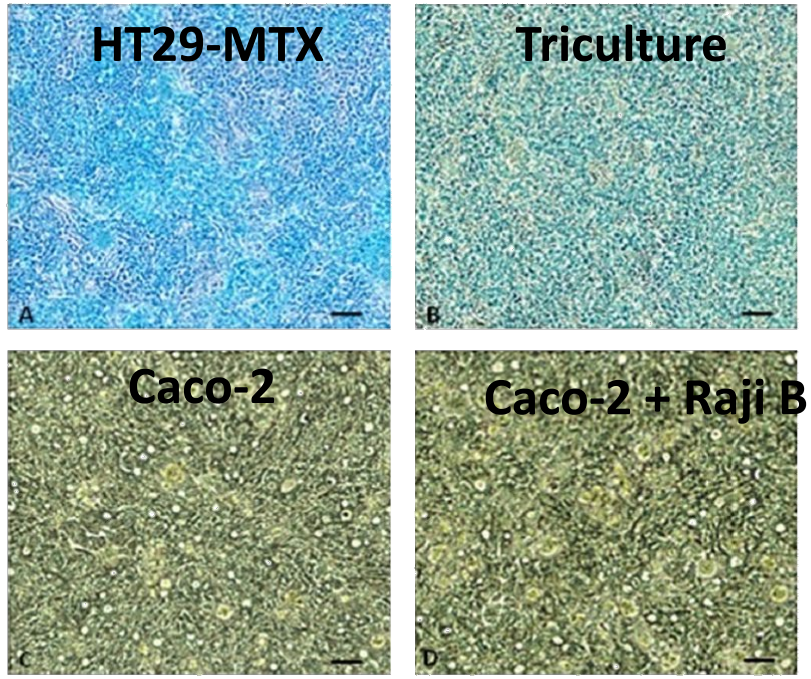
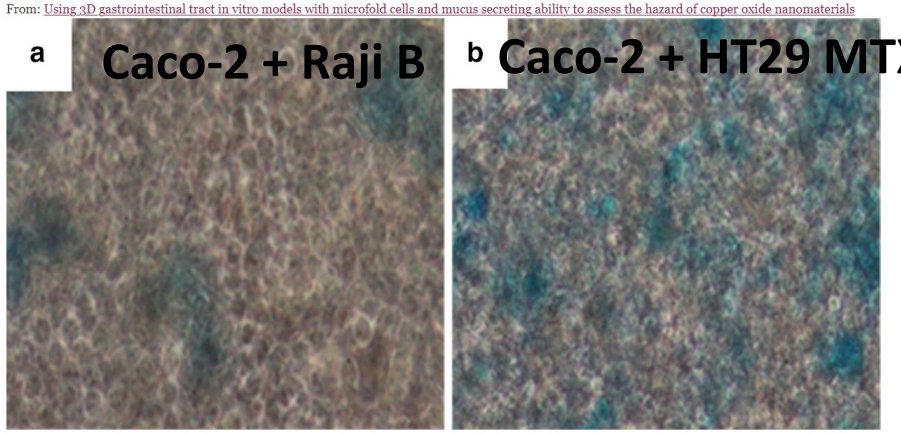


Fig. 2. Alcian Blue mucus staining. HT29-MTX cells were able to produce mucus not only in monocultures (A) but also when they grow together with Caco-2 and Raji B cells (B). The lack of staining in Caco-2 (C) and Caco-2:Raji B dual co-culture (D) confirms that mucus is only produce by HT29-MTX cells. Bar = 50 µm.

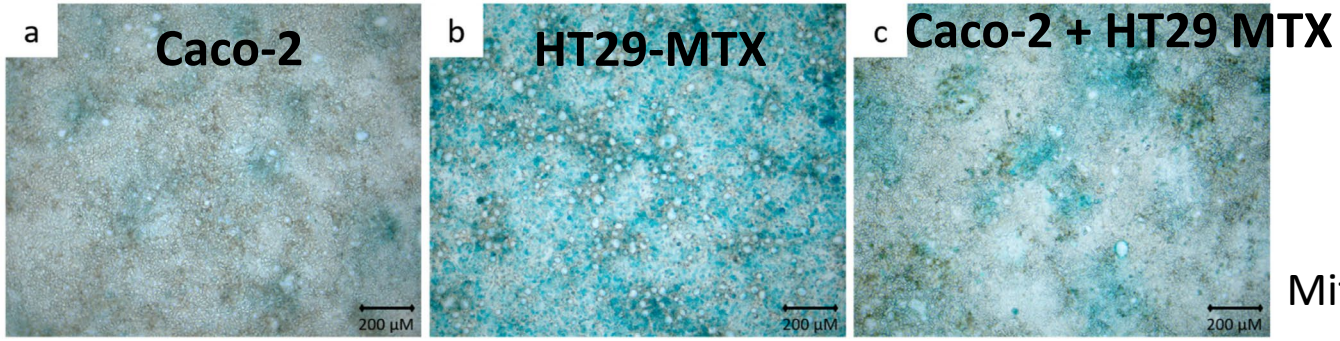
Ude et al. 2019

Fig. 2

From: Using 3D gastrointestinal tract in vitro models with microfold cells and mucus secreting ability to assess the hazard of copper oxide nanomaterials

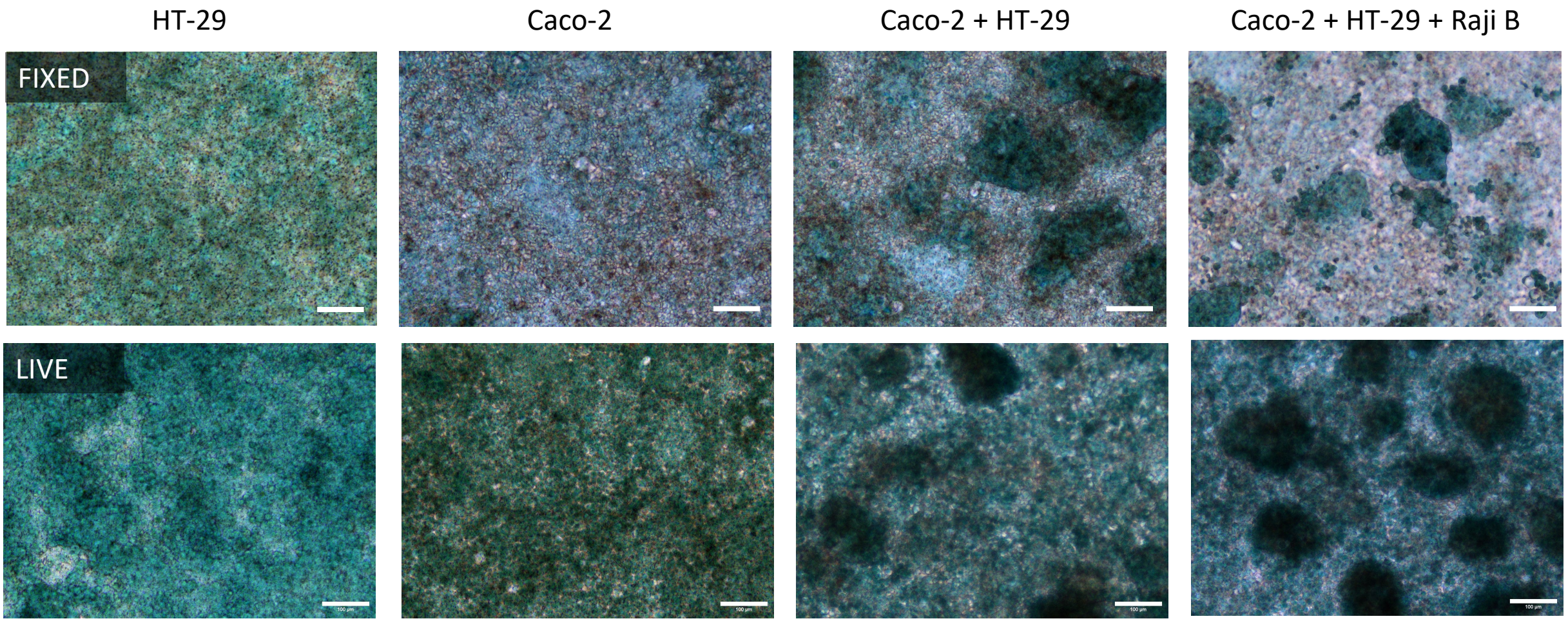


Mucus staining with Alcian Blue. The Caco-2/Raji B (**a**) and Caco-2/HT29-MTX (**b**) co-culture models were stained with Alcian blue and imaged with a ZEISS light microscope. The blue colour is mucus stained with Alcian blue. Representative images are shown



Mittag et al. 2022

Alcian Blue staining: Summary



Scale bar: 100 µm

- HT-29 seems to have a more teal blue hue than the monolayer model (darker blue).
- Caco-2 still shows a blue hue throughout.
- Differences are more evident in bi- and triculture models, where patches of blue are observed throughout.

TEER measurements – All models

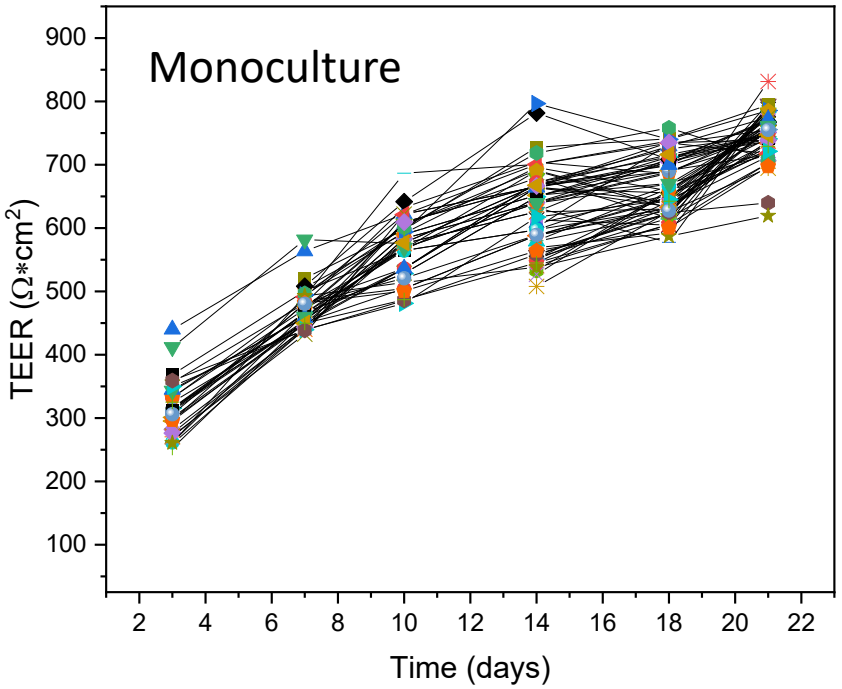
Individual measurements of five sets (SETS 4-8)
with 1 µm inserts

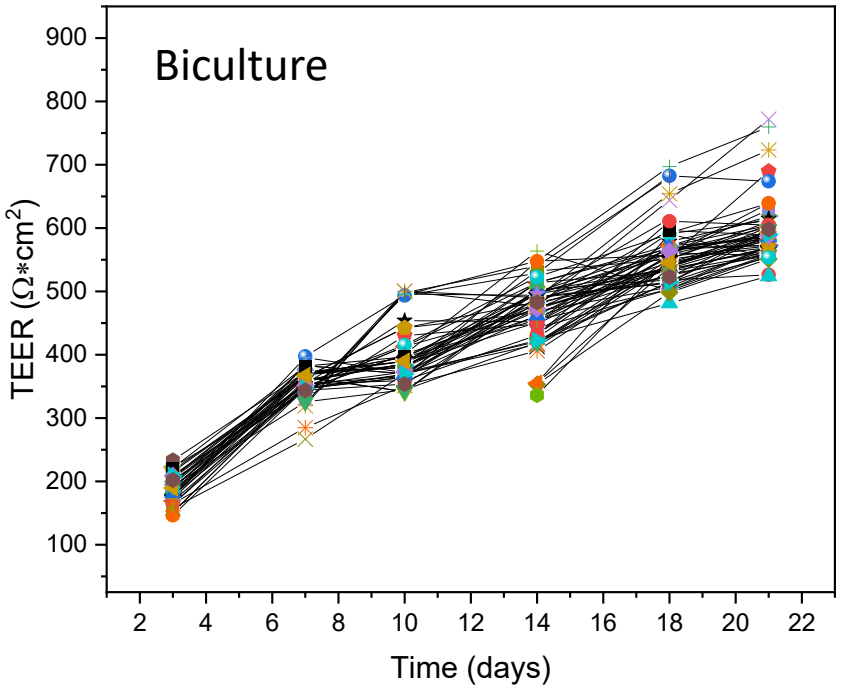
	1	2	3	4
A	Insert	Mono	HT-29	Tri
B	Insert	Mono	HT-29	Tri
C	Insert	Mono	HT-29	Tri

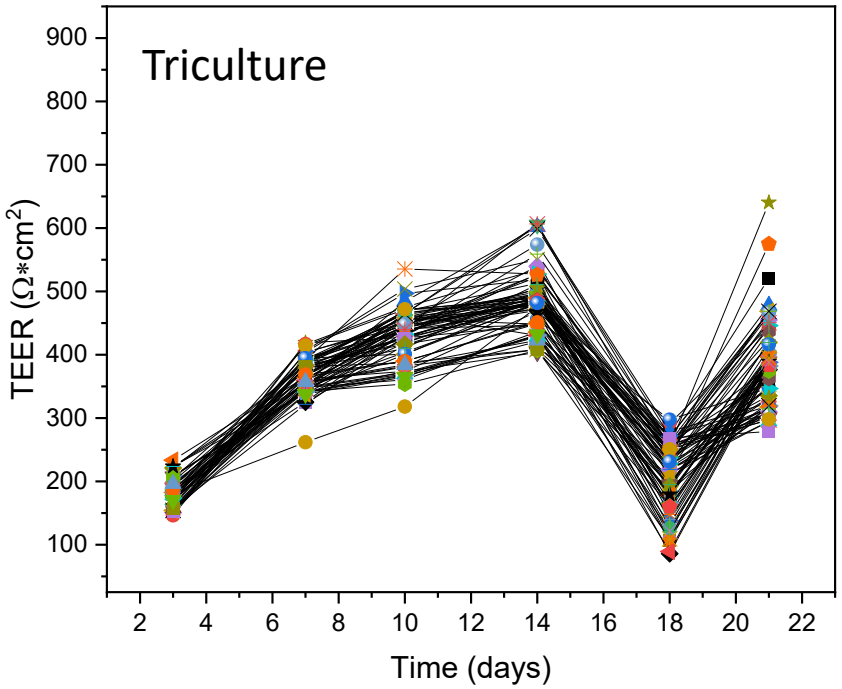
Plate 1

	1	2	3	4
A	Tri	Tri	Bi	Bi
B	Tri	Tri	Bi	Bi
C	Tri	Tri	Bi	Bi

Plate 2







TEER measurements – All models

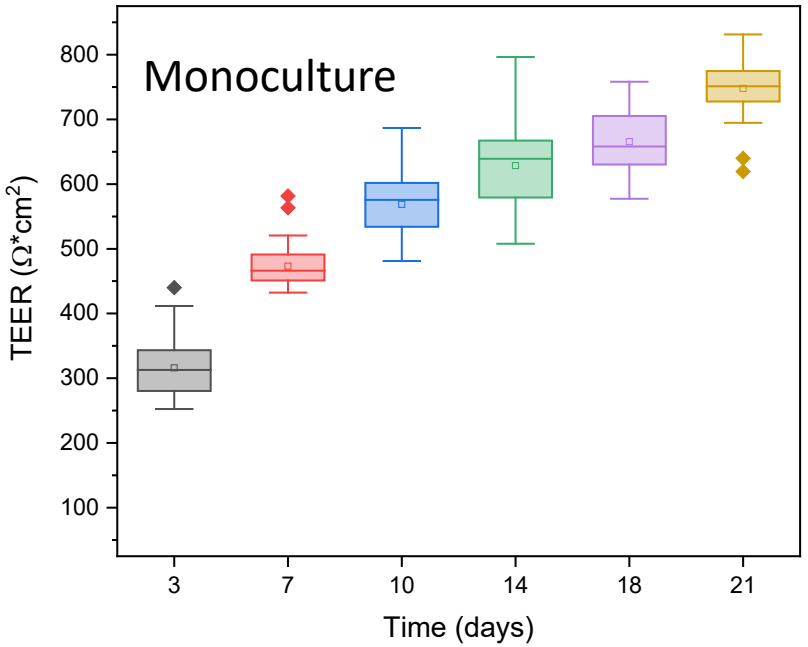
Average measurements of five sets with 1 µm inserts

	1	2	3	4
A	Insert	Mono	HT-29	Tri
B	Insert	Mono	HT-29	Tri
C	Insert	Mono	HT-29	Tri

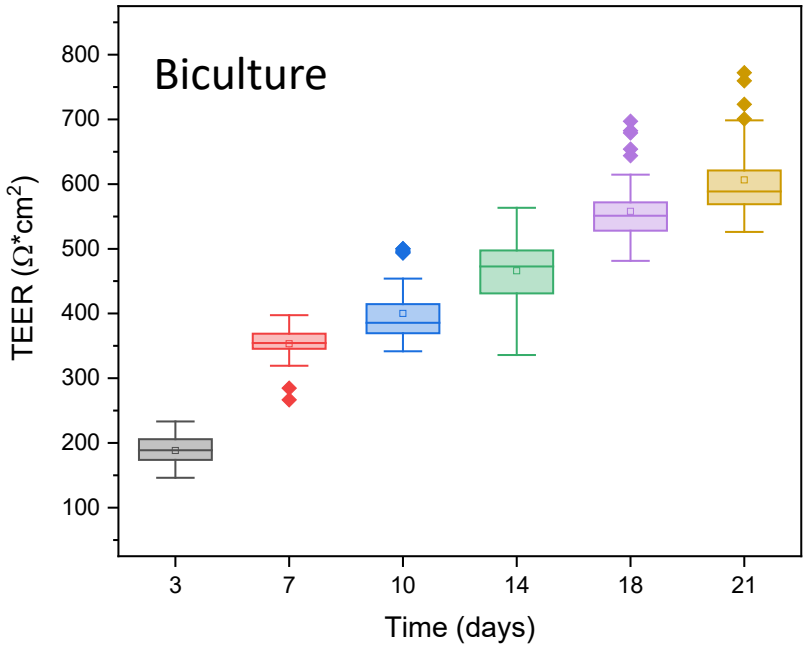
Plate 1

	1	2	3	4
A	Tri	Tri	Bi	Bi
B	Tri	Tri	Bi	Bi
C	Tri	Tri	Bi	Bi

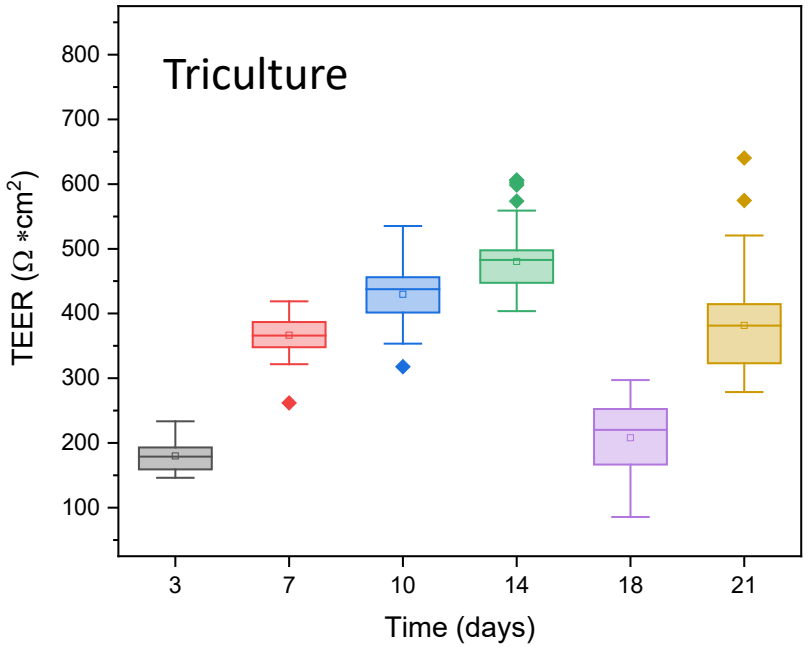
Plate 2



	N Analysis	Mean	Standard Deviation
3	30	316	43.8
7	42	473	31.4
10	42	569	46.7
14	60	629	63.0
18	60	665	46.1
21	58	748	36.6



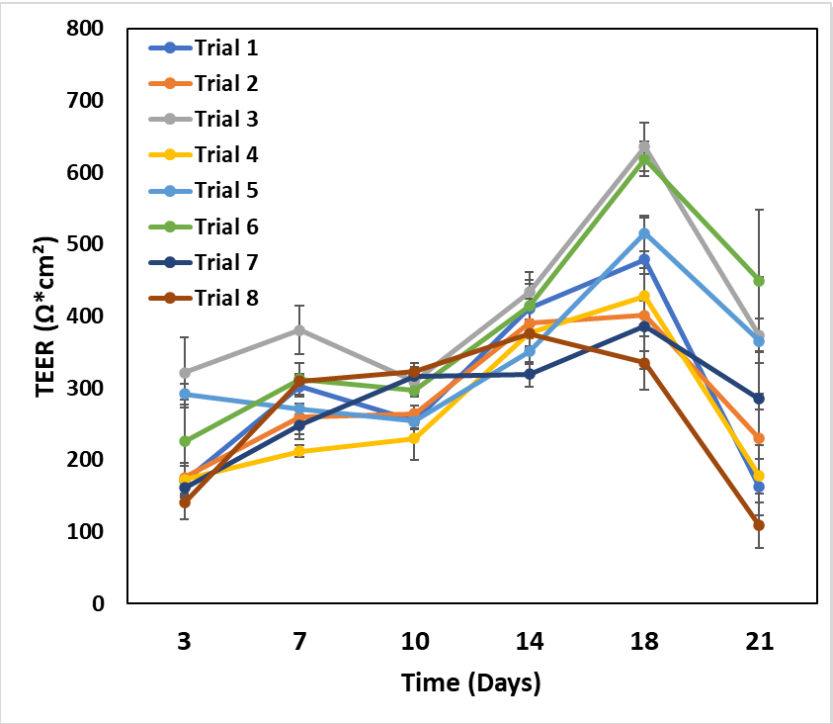
	N Analysis	Mean	Standard Deviation
3	36	188	21.8
7	44	353	25.0
10	44	400	46.5
14	56	466	49.9
18	56	558	46.1
21	54	596	49.2



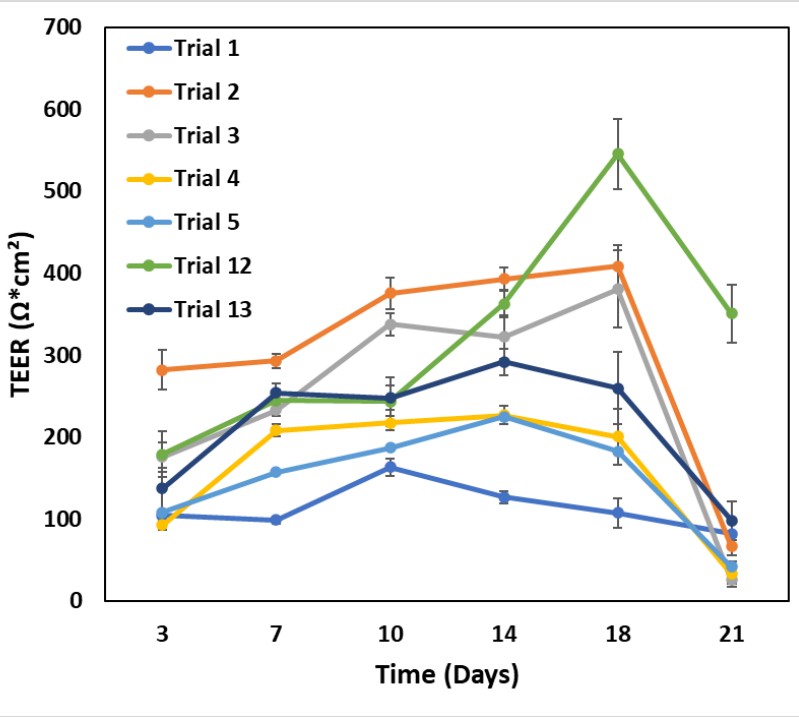
	N Analysis	Mean	Standard Deviation
3	54	180	22.1
7	72	366	26.3
10	72	430	39.6
14	84	480	46.2
18	78	208	53.2
21	76	382	65.8

TEER measurements – 2nd operator

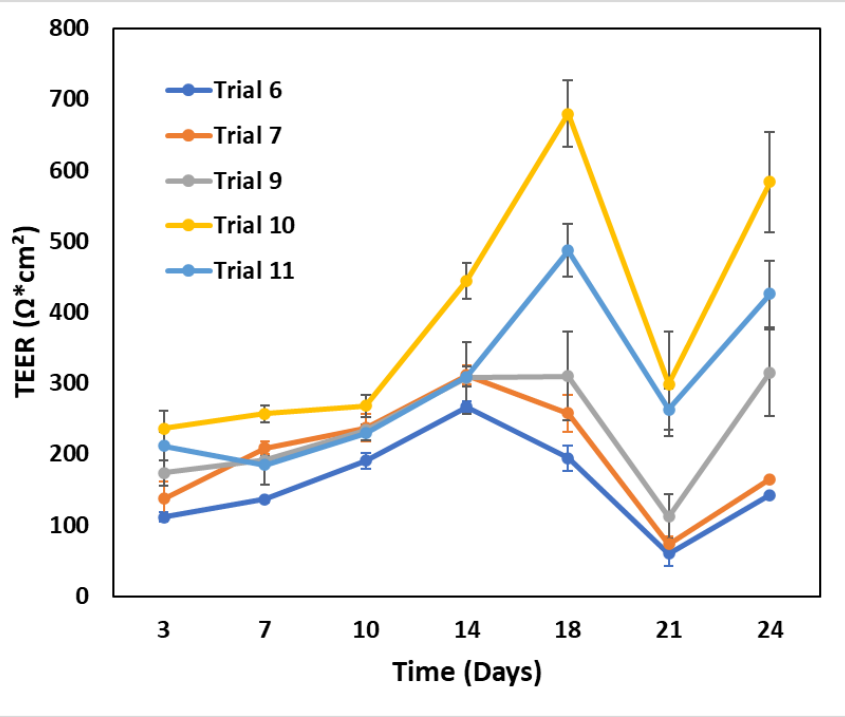
1 µm inserts



3 µm inserts



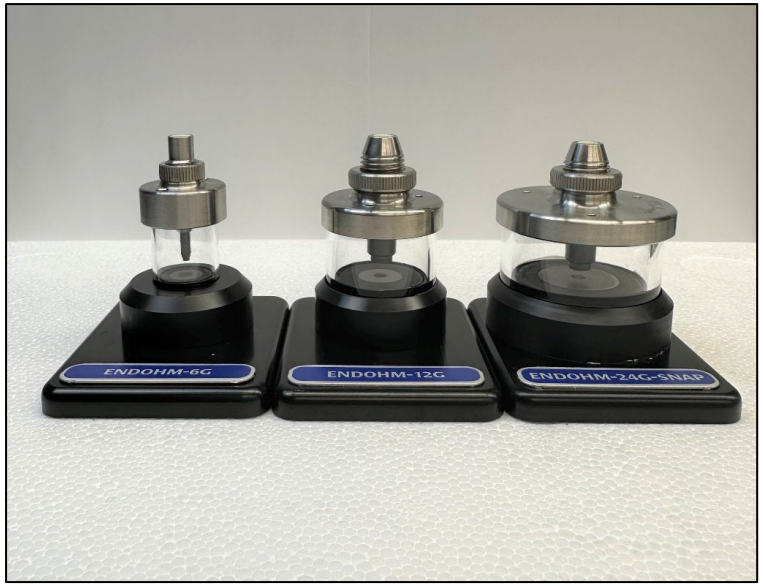
3 µm inserts extended duration



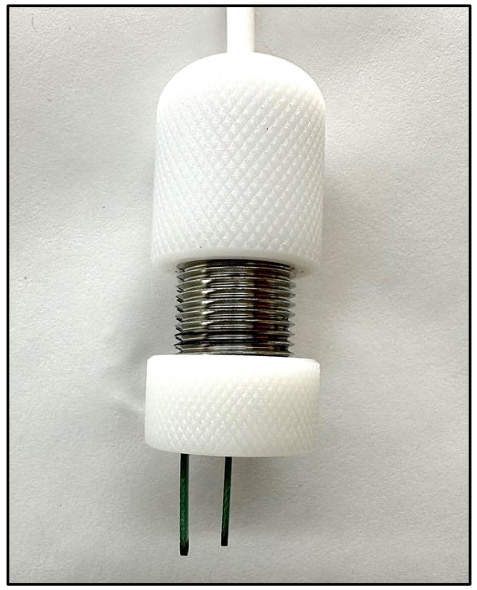
- There was not a recovery up to 21 d with either the 1 or 3 µm inserts. A longer recovery time was needed.
- Substantial variability in the final TEER values among experiments but the cause was unclear.

Comparison of three different measurement methods

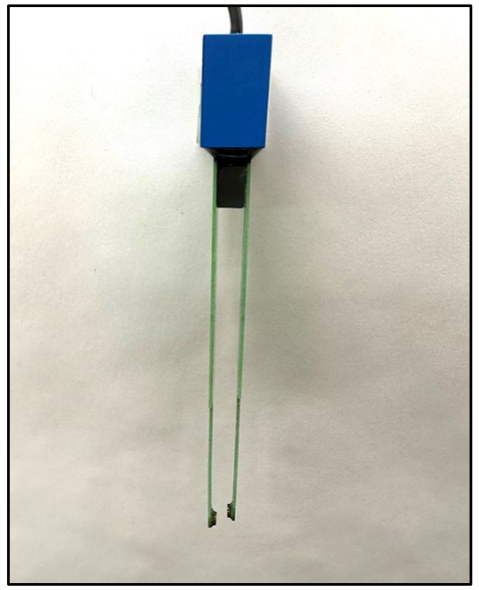
EndOhm chambers



STX4 prong



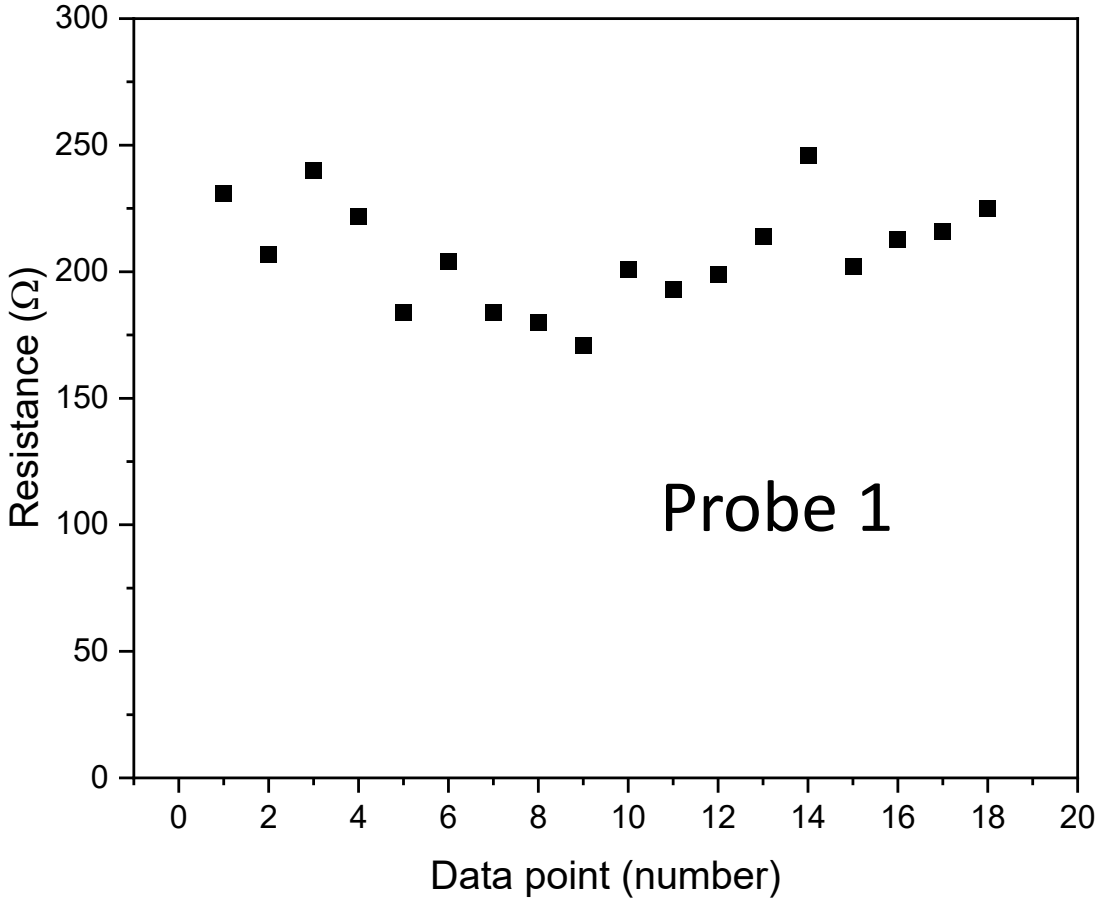
STX2 prong

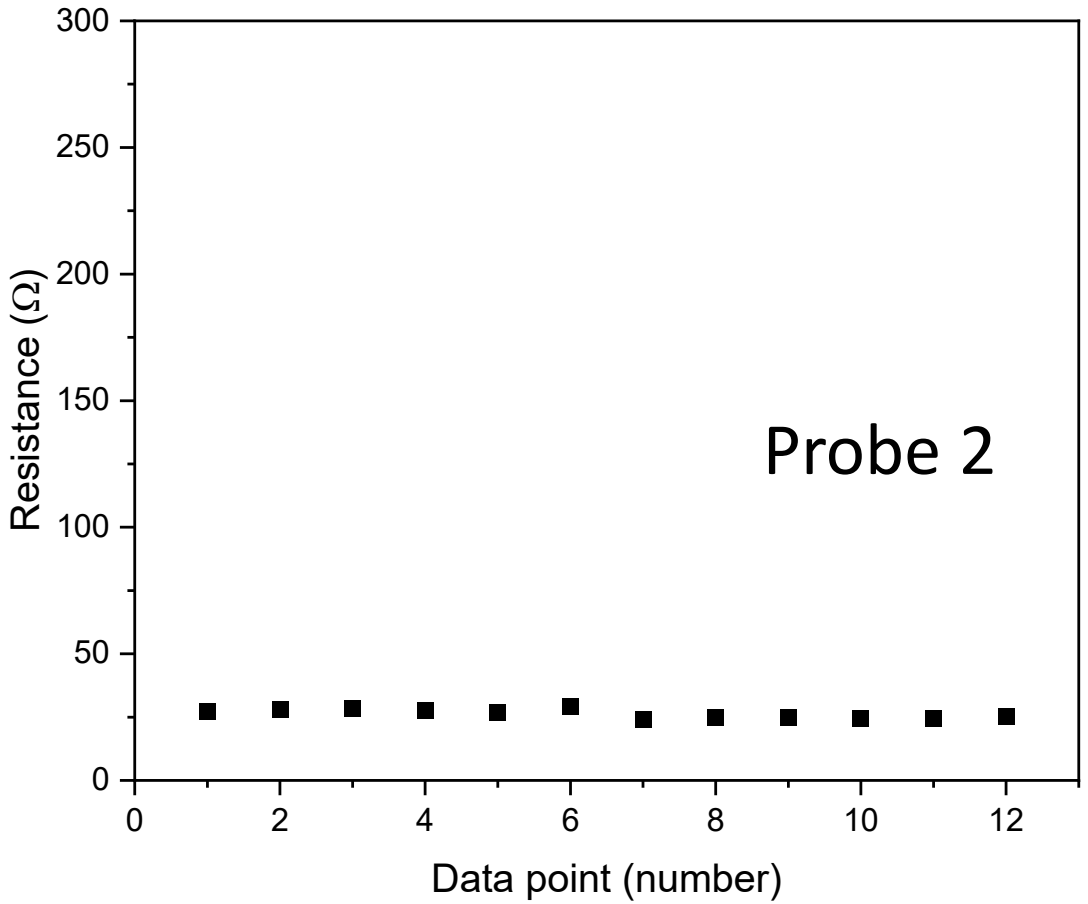


STX2 measurements were made with the Millicell instrument, while the other measurements were made with the WPI EVOM3

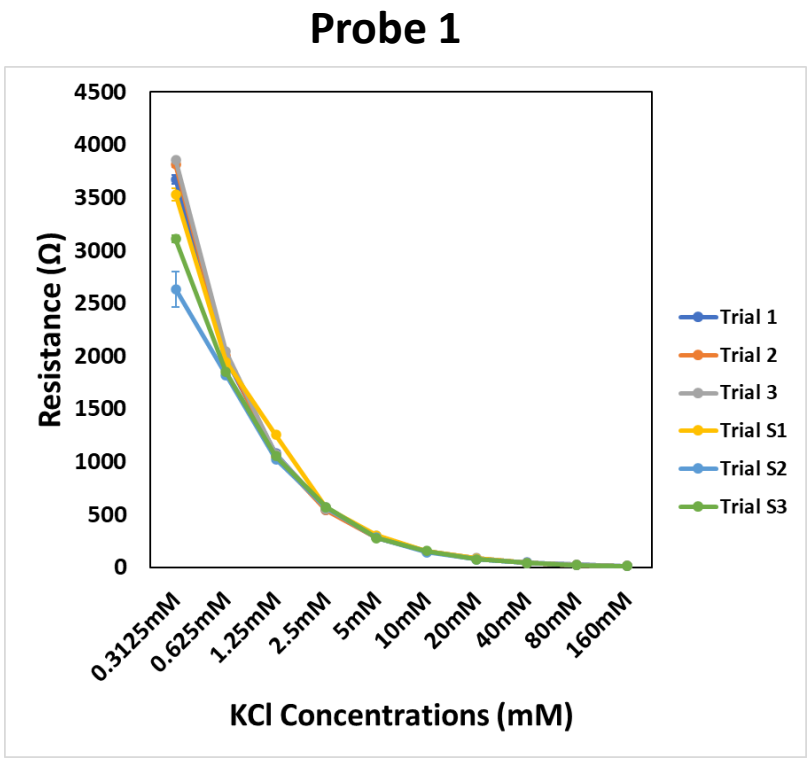
Trans-epithelial electrical resistance (TEER)

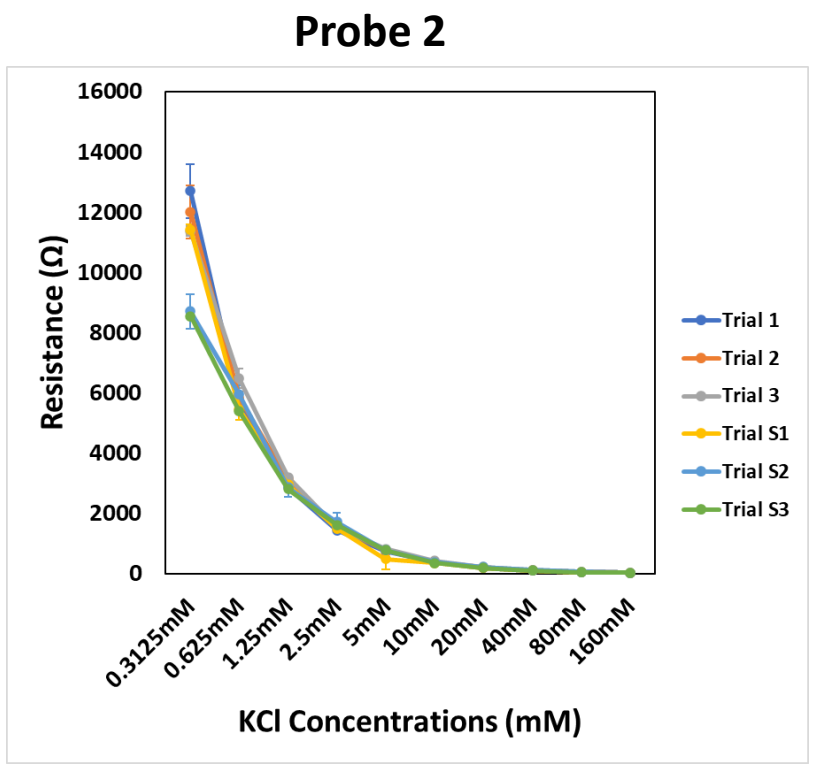
Blank measurements

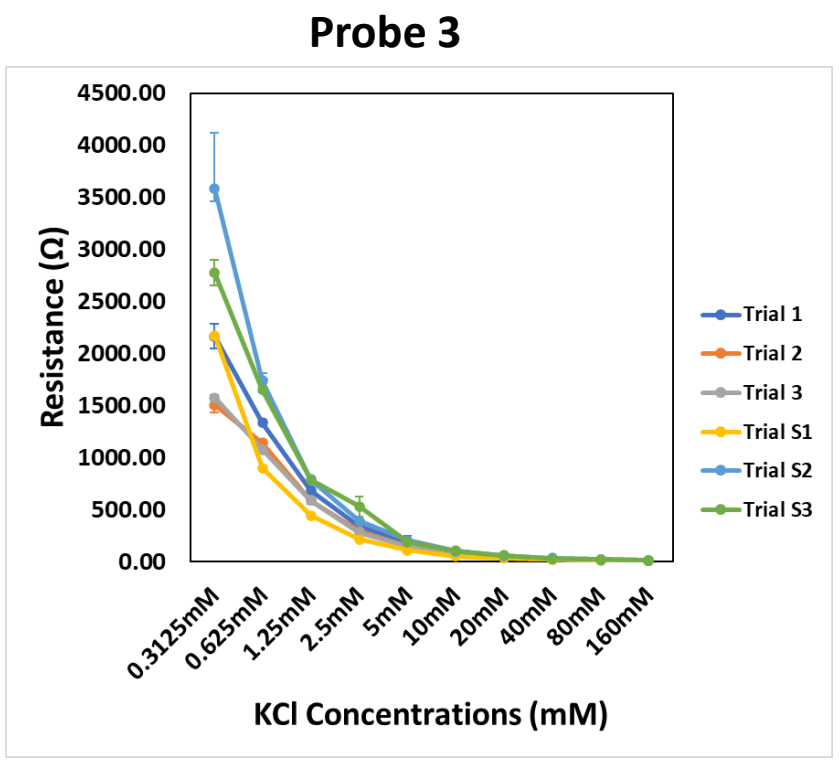




Comparing among probes and operators





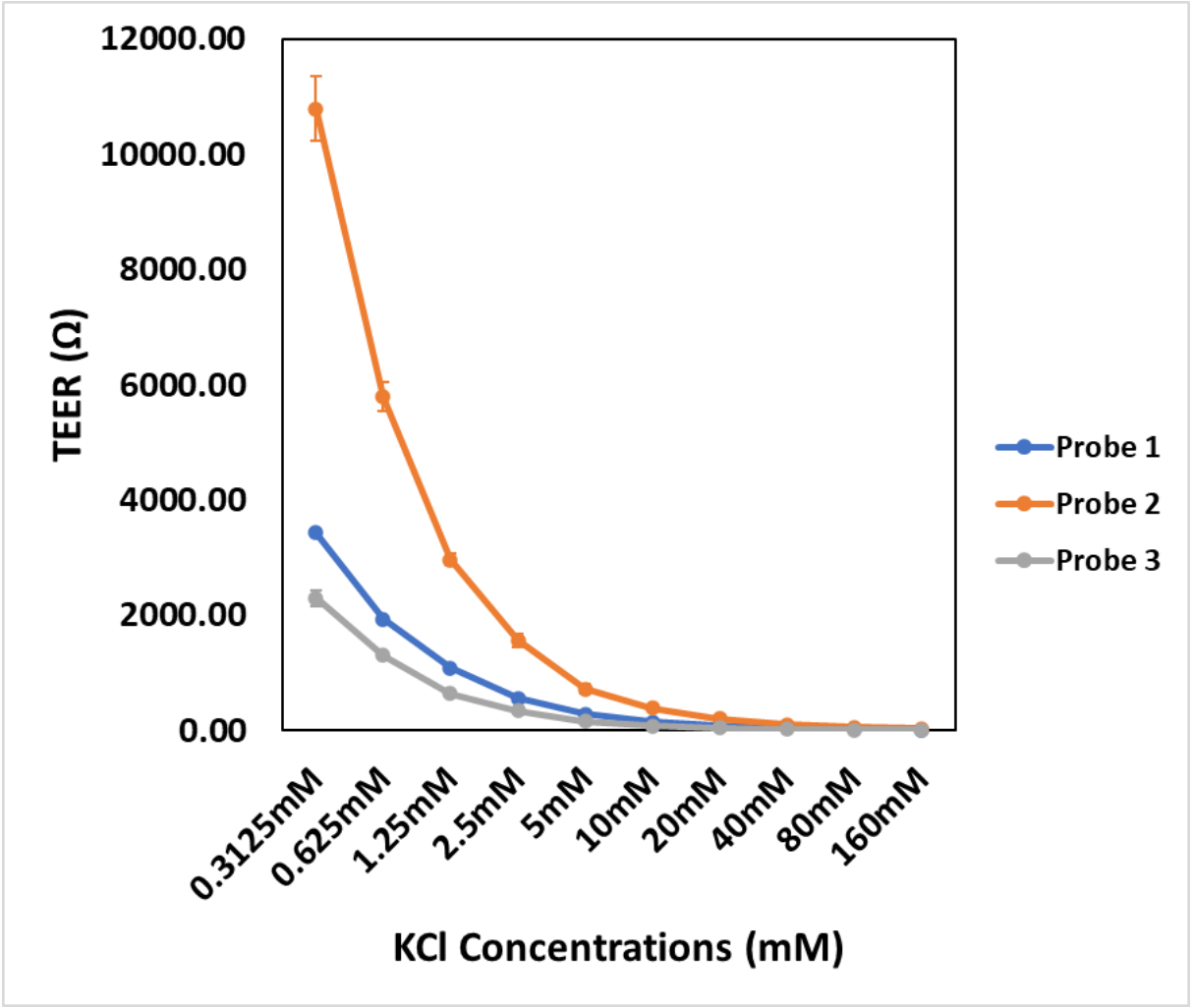


- Trials: 1, 2, and 3 and S1, S2, and S3 were performed by different operators on separate days
- Excluding the first data point, the coefficient of variation values for Probes 1, 2, and 3 ranged from 2 % to 8 %, 5 % to 16 %, and 20 % to 33 %, respectively

Comparing among systems

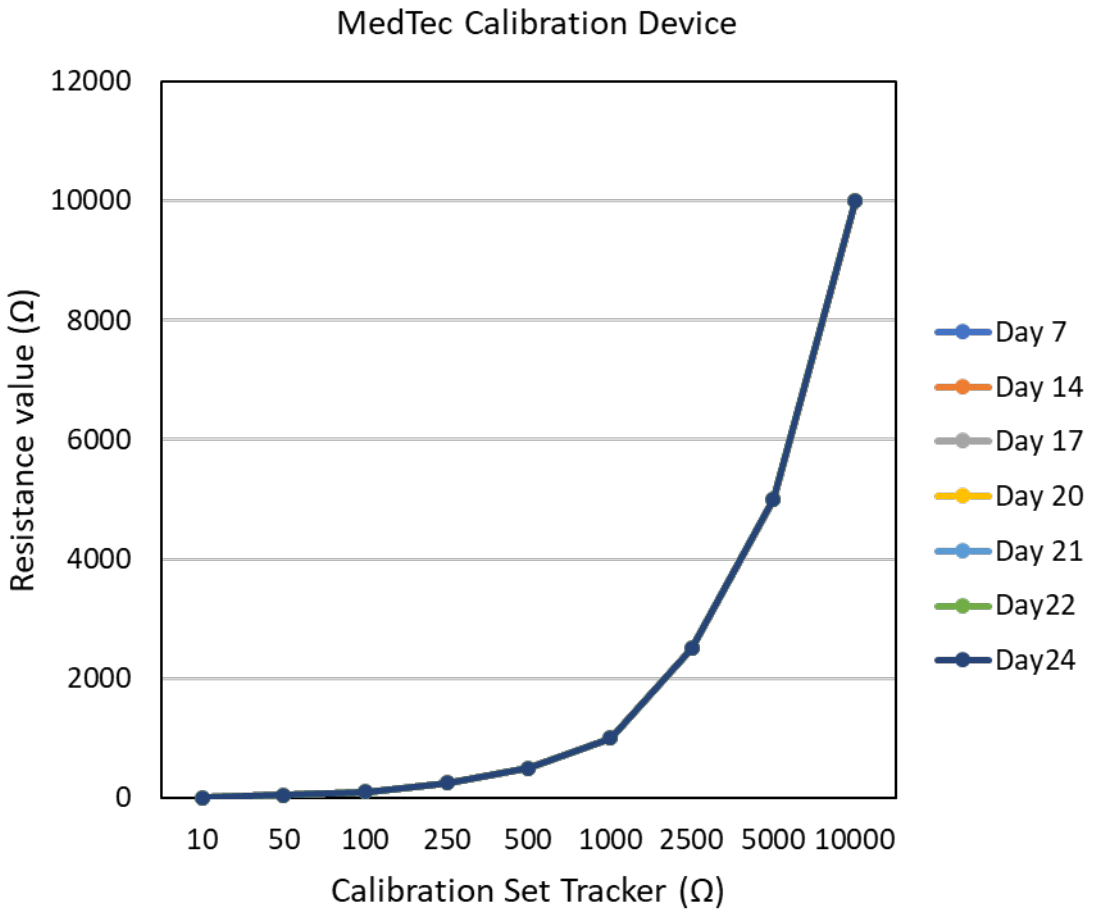
Note:

N=3 for all measurements

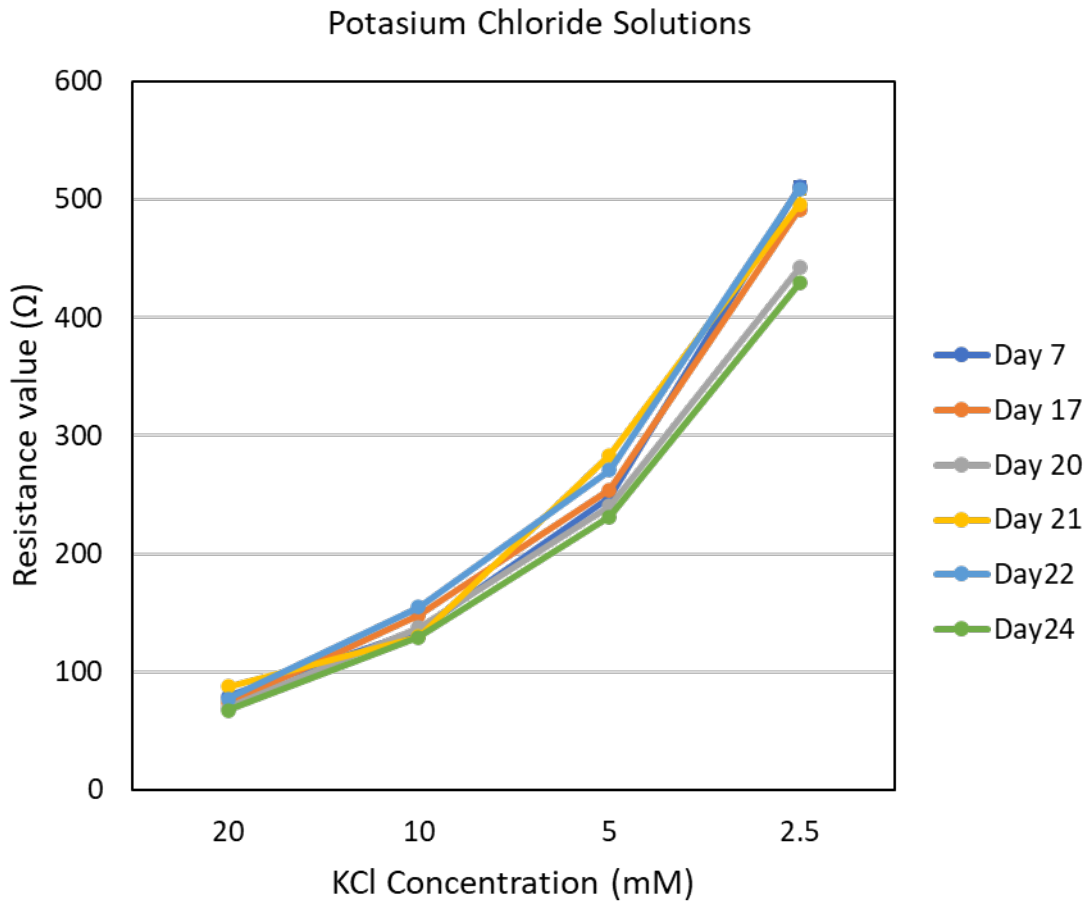


All three systems provide different values at lower concentrations (higher resistance)

Calibration control charting

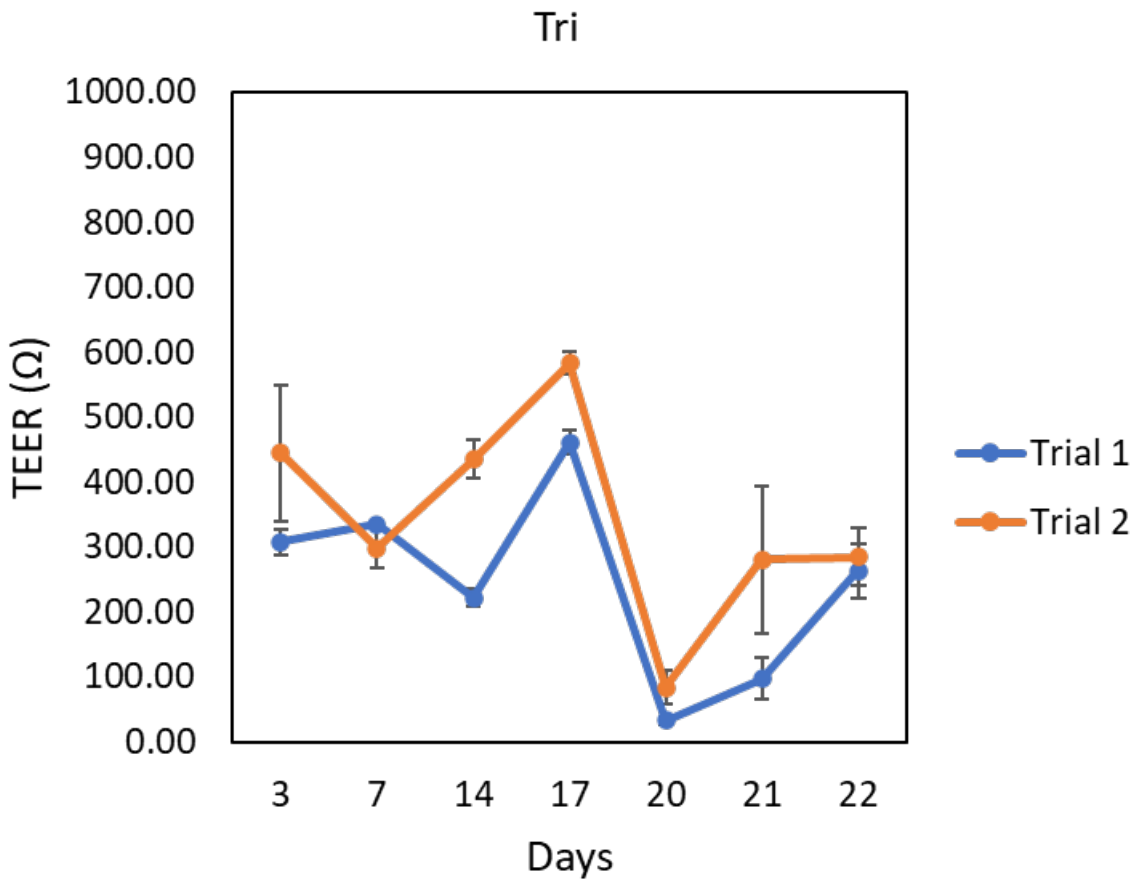


Resistance value is consistently in alignment



The COV values for the different concentrations 7 % to 9 %

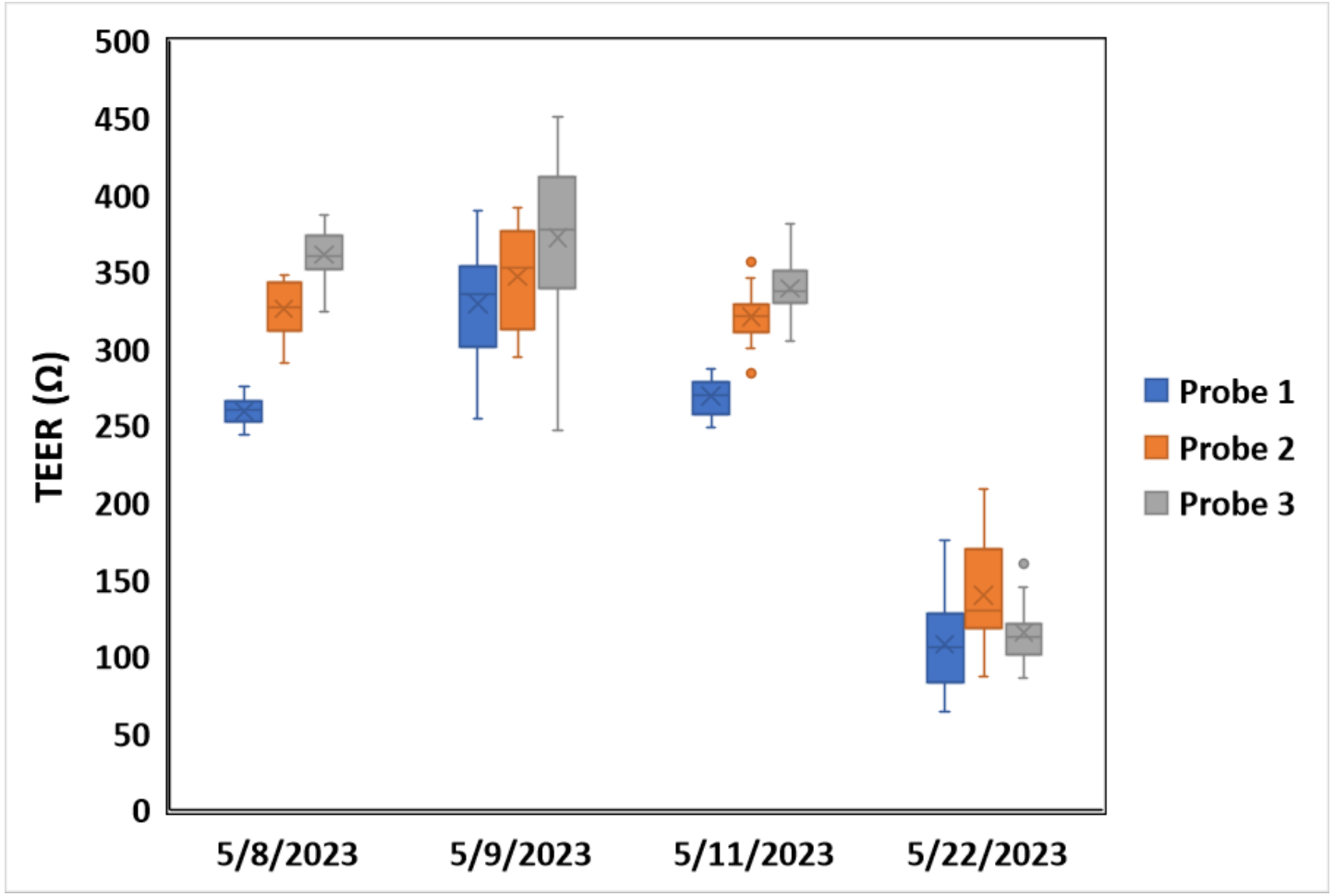
Example data showing day-to-day variability



The decreases from day 7 to 14 for Trial 1 and from day 3 to 7 for Trial 2 are suspected to be due to the TEER calibration, not a biological change

Human Gut Model Analyzed by each TEER system

n=18 samples, three replicates per sample



5/8 & 5/11 - Conducted by Operator 1

5/9 & 5/22 – Conducted by Operator 2

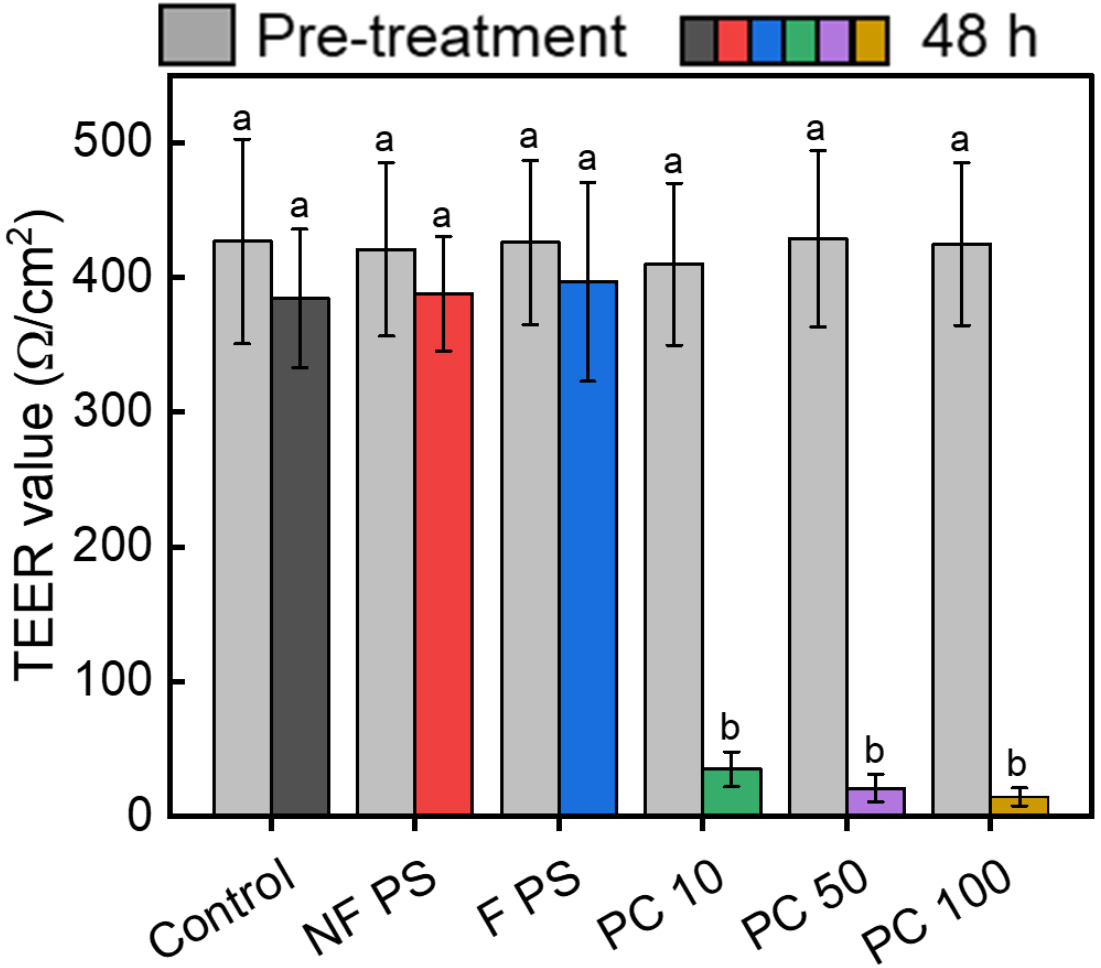
Conclusions: Operator can contribute to variability; all systems gave similar values

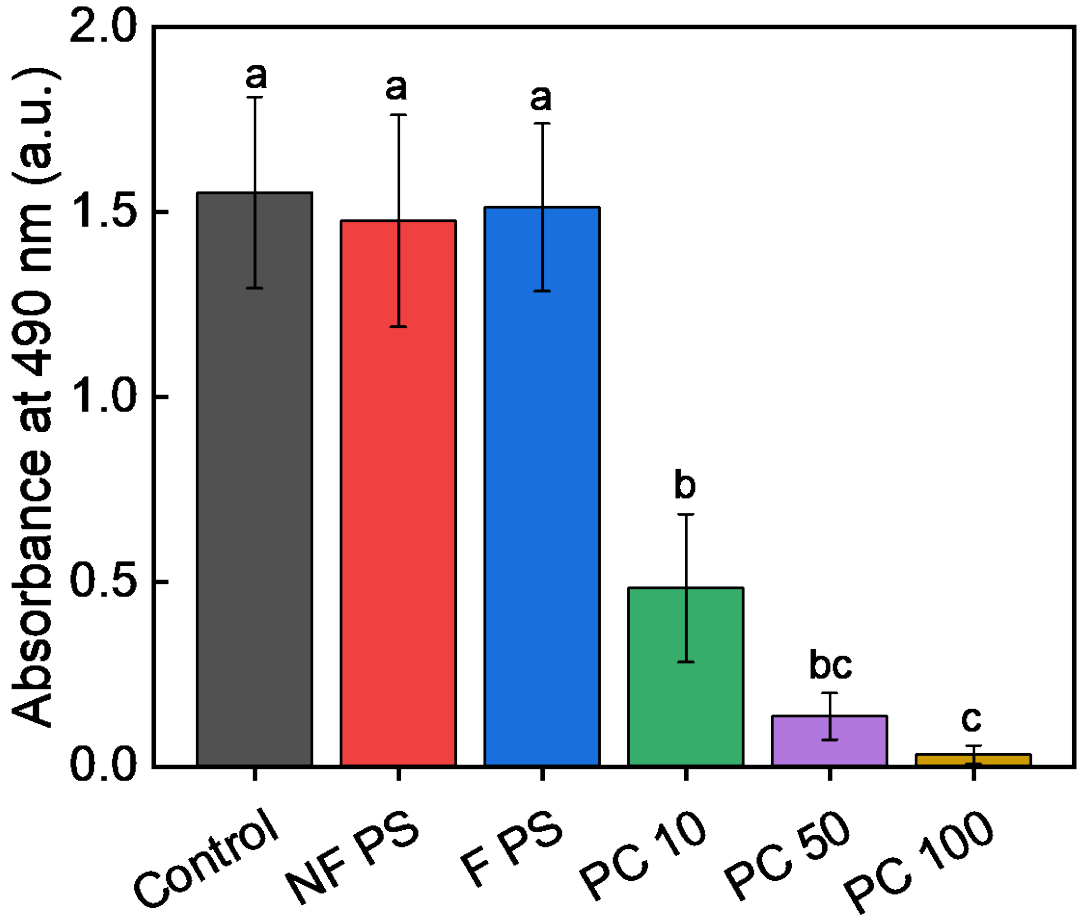
Human Gut Model Analyzed by each TEER system

	COV (%) among mean values among samples			*COV (%) of triplicate analyses per replicate*		
Date	**Probe 1**	**Probe 2**	**Probe 3**	**Probe 1**	**Probe 2**	**Probe 3**
5/8/23 3 um	5	7	8	5	5	10
5/8/23 1 um	3	5	5	2	8	7
5/9/23 1 um	11	9	14	4	8	12
5/11/23 1 um	4	5	5	4	4	5
5/22/23 1 um	28	24	19	4	8	6

n=18 samples, three replicates per sample
The higher COV values for the 5/22/23 data may be partly from the smaller TEER values as a result of Raji B addition

Viability testing





We tested fluorescence and non-fluorescence polystyrene particles (NF PS and F PS) as well as different concentrations of $CdSO_4$ (values are µM) using TEER and the MTS assay

Discussion

- TEERs measurements are challenging in part because robust guidance and protocols are not yet available
  - A particular challenge is controlling for day-to-day variability and instrument drift
- Assessment of mucus variability using microscope suggests differences between co-cultures with HT29-MTX cells and those without it, but it is challenging to quantify differences
  - ELISA methods are available but there are questions about reagent quality and stability
- MTS assay works pretty well for assessing viability changes
- M cell conversion is hard to measure
  - Electron microscopy measurements are possible but not suitable for routine usage
- The target uncertainty is unclear
  - The difference between yielding results within 20 % or a factor of 2 (or 10) is critical for assessing whether the assay is fit for purpose and the measurement assurance strategies needed
- Additional work may be needed to yield more quantitative results for M cell quantification and mucus production if higher precision is needed
- Even if better methods became available, there are not a lot of factors to adjust other than initial cell number
- Some comparison to *in vivo* methods is probably needed but it is unclear what data exists

Additional information

Alcian Blue assay

	1	2	3	4
A	Insert	Mono	HT-29	Tri
B	Insert	Mono	HT-29	Tri
C	Insert	Mono	HT-29	Tri

Plate 1

	1	2	3	4
A	Tri	Tri	Bi	Bi
B	Tri	Tri	Bi	Bi
C	Tri	Tri	Bi	Bi

Plate 2

LIVE

1. Remove medium from inserts
2. Wash cells thrice with PBS.
3. Stain with 10 mg/mL alcian blue (in 3% acetic acid) for **30 min** at RT.
4. Cells were washed with PBS five times.
5. Taken to the Cytation 5 for imaging
   Used 10x magnification

FIXED*

1. Wash inserts twice with PBS.
2. **Fix** cells with 4% formaldehyde for 25 min at RT.
3. Wash cells thrice with PBS.
4. Stain with 10 mg/mL alcian blue (in 3% acetic acid) for **30 min** at RT.
5. Cells were washed with PBS five times.
6. Taken to the Cytation 5 for imaging
   Used 10x magnification

Images were created as composites of three individual images with different colored filters.