

Mass Cytometry for Multivariate Organoid Signalling Analysis

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Method Article

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Abstract

Organoids are powerful biomimetic tissue models. Despite their increasing popularity, no existing methods are suitable for cell-type specific analysis of post-translational modification (PTM) signalling networks in organoids. Here we report a multivariate mass cytometry (MC) protocol for single-cell analysis of cell-type specific PTM signalling in organoid monocultures and organoids co-cultured with stromal and immune cells. Thiol-reactive Organoid Barcoding *in situ* (TOBis) was developed to facilitate high-throughput comparison of signalling networks between organoid cultures. Taken together, our protocol enables high-throughput multivariate PTM signalling analysis of healthy and cancerous organoids at the single-cell level.

Introduction

Organoids are self-organising 3D tissue models comprising stem and differentiated cells¹. Organoid monocultures typically contain one major cell class (e.g. epithelial) and can be co-cultured with heterotypic cell-types (e.g. mesenchymal² or immune cells³) to model cell-cell interactions *in vitro*. When compared with traditional 2D cell culture, organoids more accurately represent their parental tissue and are powerful models for studying multicellular diseases such as cancer⁴.

While culturing organoids has become increasingly widespread, methods to analyse cell-type specific phenotypes in organoids are limited. Mass cytometry (MC, also known as cytometry time-of-flight (CyTOF)) uses heavy metal-conjugated antibodies to measure >35 proteins in single cells⁵. Although MC is traditionally used for high-dimensional immunophenotyping, MC can also measure PTMs in heterocellular systems (e.g. peripheral blood mononuclear cells (PBMCs)⁶ and tissue⁷). Here we report a protocol to perform cell-type specific analysis of post-translational modification (PTM) signalling networks in organoids and organoid co-cultures using MC.

In addition to high dimensional single-cell PTM measurements, a major advantage of MC is its ability to perform multiplexed barcoding of experimental variables. Unfortunately, commercially available Palladium-based barcodes cannot bind organoids *in situ* as they react with Matrigel proteins commonly used in organoid culture. This means organoids must be removed from Matrigel and dissociated separately before using Pd barcoding. To overcome this, we developed Thiol-reactive Organoid Barcoding *in situ* (TOBis) to enable high-throughput multivariate single-cell organoid signalling analysis in a single tube.

Taken together, our protocol demonstrates how traditional MC workflows can be modified to measure cell-type and cell-state specific signal transduction in organoids in a high-throughput multiplexed manner (Figure 1).

Reagents

Murine Small Intestinal Organoid Culture⁸

Growth Factor Reduced Matrigel (Corning, Cat# 354230)

Advanced DMEM/F-12 (Thermo, Cat# 12634010)

L-Glutathione (Sigma, Cat# G6529)

N-Acetyl-L-Cysteine (Sigma, Cat# A9165)

HEPES (Sigma, Cat# H3375)

B-27 Supplement (Thermo, Cat# 17504044)

N-2 Supplement (Thermo, Cat# 17502048)

Murine EGF (Thermo, Cat# PMG8041)

Murine Noggin (Peprotech, Cat# 250-38)

Murine R-Spondin-1 (Peprotech, Cat# 315-32)

HyClone™ Penicillin Streptomycin Solution (Thermo, Cat# SV30010)

Mass Cytometry Analysis

¹²⁷5-Iodo-2'-deoxyuridine (¹²⁷IdU) (Fluidigm, Cat# 201127)

Protease Inhibitor Cocktail (Sigma, Cat# P8340)

PhosSTOP™ (Sigma, Cat# 4906845001)

Paraformaldehyde solution, 4% in PBS (Thermo, Cat# J19943K2)

^{194/8}Cisplatin (Fluidigm, Cat# 201194/8)

Dispase II (Thermo, Cat# 17105041)

Collagenase IV (Thermo, Cat# 17104019)

DNase I (Sigma, Cat# DN25)

¹⁹⁶Cisplatin (a gift from Fluidigm)

TeMal (¹²⁴Te, ¹²⁶Te, ¹²⁸Te, ¹³⁰Te) (a gift from Prof. Mark Nitz, University of Toronto)⁹

Cell Staining Buffer (Fluidigm, Cat# 201068)

Maxpar® X8 Multimetal Labeling Kit (Fluidigm, Cat# 201300)

Triton X-100 (Sigma, Cat# T8787)

Methanol (Fisher, Cat# 10675112)

Pierce™ 16% Formaldehyde (w/v), Methanol-free (Pierce, Cat# 28906)

Cell-ID™ Intercalator-Ir (Fluidigm, Cat# 201192A)

EQ™ Four Element Calibration Beads (Fluidigm, Cat# 201078)

Rare-Earth Metal Conjugated Antibodies

We advise users to develop custom heavy-metal conjugated antibody panels specifically for their biological questions. In our experience, cell-state (e.g. proliferating, quiescent, and apoptotic) has a huge influence on PTM signalling. We therefore strongly advise users to include cell-cycle (e.g. pRB [S807/S811], Cyclin B1, and pHistone H3 [S28]) and apoptosis (e.g. cCaspase3 [D175]) markers in their panels. Particular care should also be taken to validate cell-type specific antibodies that have not been used in MC before. The antibody panels used when developing this protocol can be found in Qin *et al.*, bioRxiv, 2019¹⁰.

Thiol-reactive Organoid Barcoding *in situ* (TOBis)

Debarcoding efficiency is heavily dependent on aligned Te and Pt signal intensities. We advise TeMal (¹²⁴Te, ¹²⁶Te, ¹²⁸Te, and ¹³⁰Te) and Cisplatin (¹⁹⁶Pt and ¹⁹⁸Pt) isotopologs to be carefully titrated to achieve a common 'on' median intensity at $\sim 5 \times 10^2$ and 'off' median intensity of $< 1 \times 10^1$ (Figure 2). TeMals should be used between 500-1000 nM and Cisplatin between 10-20 nM.

Equipment

gentleMACS C-Tube (Miltenyi, Cat# 130-096-334)

gentleMACS Octo Dissociator (with Heaters) (Miltenyi, Cat# 130-096-427)

Helios Mass Cytometer (Fluidigm)

Procedure

S-Phase Cell Labelling

1) Add ^{127}IdU directly to culture media to a final concentration of 25 μM . Gently rotate the plate, and incubate for 25 mins at 37 °C, 5 % CO_2 .

Phosphatase & Protease Inhibitor Treatment

2) Add the protease inhibitor cocktail (100 \times) and PhosSTOP (40 \times) directly to culture media, rotate the plate, and incubate for 5 mins at 37 °C, 5 % CO_2 .

Fixation

3) Remove culture media quickly. Add pre-warmed 4% PFA into each well with care taken not to disrupt Matrigel droplets. Incubate for 60 mins at 37 °C, 5 % CO_2 .

4) Remove PFA solution. Wash the cells with PBS on a rocker for 10 mins at room temperature. Repeat wash.

Optional: Stopping point – The fixed cells can be kept in PBS at 4 °C for up to four weeks.

Live / Dead Discrimination

5) Remove PBS. Add 0.25 μM $^{194/8}\text{Cisplatin}$ / PBS solution to each well and incubate for 10-15 mins on a rocker at room temperature.

6) Remove the $^{194/8}\text{Cisplatin}$ solution. Wash the cells with PBS on a rocker for 10 mins at room temperature. Repeat wash.

Optional: If barcoding multiple organoid samples continue to step 7. If only one organoid culture condition is being analysed, skip to step 10.

Thiol-reactive Organoid Barcoding *in situ* (TOBis) (Optional)

7) Resuspend TOBis barcodes in PBS and add them to corresponding organoid samples. Incubate the cells overnight at 4 °C.

8) Remove the barcoding solutions and wash the cells with 2 mM Glutathione / CSB for 10 mins on a rocker at room temperature. Repeat wash twice.

9) Wash the cells with PBS for 10 mins on a rocker at room temperature. Repeat wash.

Single-Cell Dissociation

10) Make up a dissociation solution of fresh 0.5 mg/mL Dispase II, 0.2 mg/mL Collagenase IV, and 0.2 mg/mL DNase I in PBS.

- 11) Remove PBS from the wells and add the dissociation solution.
- 12) Scrape Matrigel droplets and transfer the cells with dissociation solution to a gentleMACS C-tube. Top up the dissociation solution to 5 mL / C-tube.
- 13) Dissociate the organoids into single cells using a gentleMACS Octo Dissociator.

Custom Program:

- Set Heater temperature: 37 °C

- Forward spin at 20 rpm for 2 mins

- Backward spin at 20 rpm for 2 mins

- Loop 15X:

* Forward spin at 1500 rpm for 2 secs

* Backward spin at 1500 rpm for 2 secs

* Forward spin at 50 rpm for 3 mins

- End loop

14) After confirmation of sufficient dissociation, centrifuge the C-tubes at 800Xg for 1 min to collect the cells.

15) Transfer all cells and solution to a polypropylene FACS tube.

16) Centrifuge the cells at 800Xg for 5 mins and discard the supernatant.

17) Wash the cells with 2 mL CSB, centrifuge at 800Xg for 5 mins, and discard the supernatant. Repeat wash.

Optional: Stopping point – The fixed cells can be kept in CSB at 4 °C for up to four weeks.

18) Centrifuge the cells, discard the supernatant, and resuspend the cells in 50 µL CSB.

Extracellular Stain

19) Prepare extracellular antibody cocktail in CSB (total volume up to 50 µL).

20) Add the extracellular antibody cocktail to the cells, and incubate for 30 mins on a rocker at room temperature. Mix cells every 10 mins to avoid cells pelleting.

21) Add 2 mL CSB to the cells, centrifuge at 800 Xg for 5 mins, and discard the supernatant.

Permeabilisation

- 22) Resuspend the cells in 1 mL of 0.1 % Triton X-100 / PBS, gently vortex, and incubate for 30 mins on a rocker at room temperature. Mix cells every 10 mins to avoid cells pelleting.
- 23) Add 2 mL CSB to the cells, centrifuge at 800 ×g for 5 mins, and discard the supernatant. Repeat wash and remove supernatant.
- 24) Place the cells on ice for 1 min. Resuspend the cells in 1 mL of ice-cold 50% Methanol / PBS (store at -20 °C until use) and incubate for 10 mins on ice.
- 25) Add 2 mL CSB to the cells, centrifuge at 800 ×g for 5 mins, and discard the supernatant. Repeat wash.
- 26) Resuspend the cells in 50 µL CSB.

Intracellular Stain

- 27) Prepare intracellular antibody cocktail in CSB.
- 28) Add the intracellular antibody cocktail to the cells, and incubate for 30 mins on a rocker at room temperature. Mix cells every 10 mins to avoid cells pelleting.
- 29) Add 2 mL CSB to the cells, centrifuge at 800×g for 5 mins, and discard the supernatant.

Post-Staining Fixation

- 30) Add 1 mL 1.6% formaldehyde (FA) / PBS solution made fresh from 16% FA to the cells.
- 31) Incubate the cells on a rocker for 10 mins at room temperature.
- 32) Add 1 mL CSB to the cells, centrifuge at 800×g for 5 mins, and discard the supernatant.

Optional: Stopping point – The fixed cells can be kept in CSB at 4 °C for up to one week.

DNA Intercalation

- 33) Dilute 0.75 µL Cell-ID Intercalator-Ir in 1 mL Fix & Perm Buffer.
- 34) Resuspend the cells in the intercalation buffer, gently vortex, and incubate for 1 hr at room temperature or overnight at 4 °C.

Mass Cytometry Data Acquisition

- 35) Centrifuge the cells at 800×g for 5 mins, and discard the supernatant.

- 36) Wash the cells with 2 mL CSB, centrifuge at $800\times g$ for 5 mins, and discard the supernatant. Repeat wash.
- 37) Wash the cells with 2 mL MaxPar Water, centrifuge at $800\times g$ for 5 mins, and discard the supernatant.
- 38) Resuspend cells in 1 mL of MaxPar Water, filter through a $35\ \mu\text{m}$ cell strainer ($70\ \mu\text{m}$ when the culture contains fibroblasts), and count the cells.
- 39) Dilute cells to $\sim 0.8 - 1 \times 10^6 / \text{mL}$ in MaxPar Water.
- 40) Add EQ Beads to the cells at a volumetric ratio of 1:5.
- 41) Add EDTA to the cells to a final concentration of 2 mM.
- 42) Analyse the sample on a Helios mass cytometer. (We advise using the “Super Sampler” (Victorian Airships) to load organoid cells into the Helios.)

Troubleshooting

Organoids are not dissociated properly.

In order for organoids to be sufficiently dissociated with the gentleMACS Octo Dissociator, we suggest users not to overload the gentleMACS C-Tubes. In our experience, up to $\sim 5 \times 10^6$ cells per C-Tube can be dissociated sufficiently using our custom dissociation program. We also recommend users to prepare fresh dissociation solution before each use.

Antibody staining is not working.

Antibody panels for MC experiments need to be carefully designed and titrated in accordance with known monoisotopic impurities¹¹ and antigen abundance. We also encourage users to test alternative fixation and permeabilisation conditions for their specific experimental system.

Cells are lost during staining.

Cell loss is inevitable during staining due to the multiple washing steps, and it is more striking with fewer cells. We recommend users to start with sufficient materials, barcode the cells, and pool different conditions as early as possible during the protocol (that is part of the motivation of the development of TOB*is*). In addition, during optimisation we observed that coating polypropylene FACS tubes with CSB prior to centrifugation of cells resuspended in PBS also facilitates the cells to spin down properly and thereby reduces cell loss.

Time Taken

The pre-treatment and fixation of organoids takes around 2 hours. Live / dead discrimination staining takes around 0.5 hour. TOB*is* barcoding can be performed in 1-2 hours but an overnight incubation will give superior debarcoding efficiencies. Depending on the number of samples, single-cell dissociation takes 2 to 2.5 hours. The MC staining protocol (extracellular staining, permeabilisation, and intracellular staining) takes around 4 hours. DNA intercalation is performed for 1 hour at room temperature or overnight at 4 °C. The MC run takes 1 to 2 hours depending on the number of cells to be analysed.

Anticipated Results

The data generated from the workflow reported here is single-cell measurements of up to 50 channels (cell-type identification antibodies, cell-state antibodies / probes, and PTM antibodies etc.) across multiple organoid culture conditions. TOB*is* barcoded conditions can be resolved into individual FCS files using: <https://github.com/zunderlab/single-cell-debarcoder>. All FCS files can be uploaded to the Cytobank platform (<http://www.cytobank.org/>) and proceed to single-cell signalling analysis¹⁰. All selection of tools to analyse organoid CyTOF data can be found at: <https://github.com/TAPE-Lab>.

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Figures

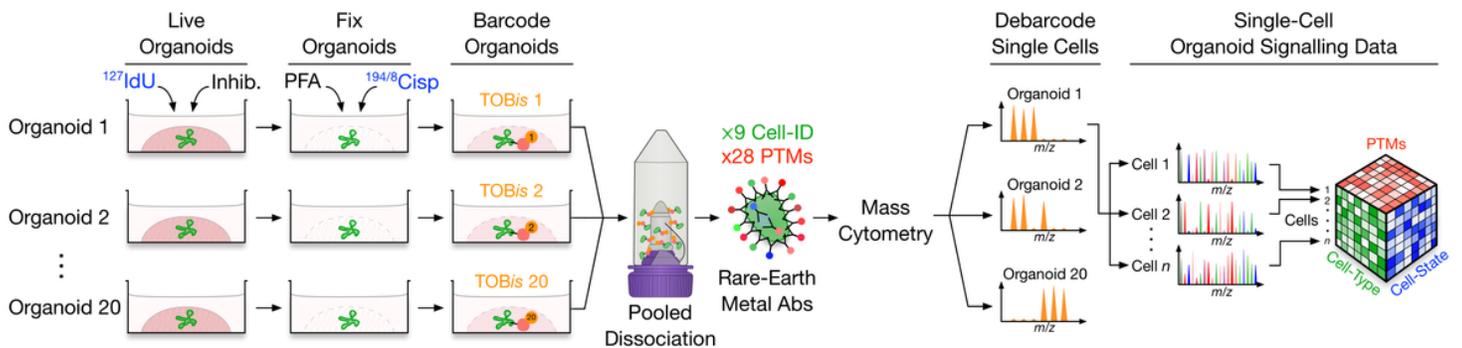


Figure 1

Experimental workflow. Live organoids are pulsed with IdU to label S-phase cells, treated with protease / phosphatase inhibitors and fixed with PFA to preserve post translational modification (PTM) signals, and stained with Cisplatin to label dead cells. Fixed organoids are barcoded using Thiol-reactive Organoid Barcoding in situ (TOBis), pooled for dissociation, stained with rare-earth metal-conjugated antibodies, and analysed by single-cell mass cytometry (MC). The resulting dataset contains integrated cell-type, cell-state, and PTM signalling information from multiple organoid culture conditions.

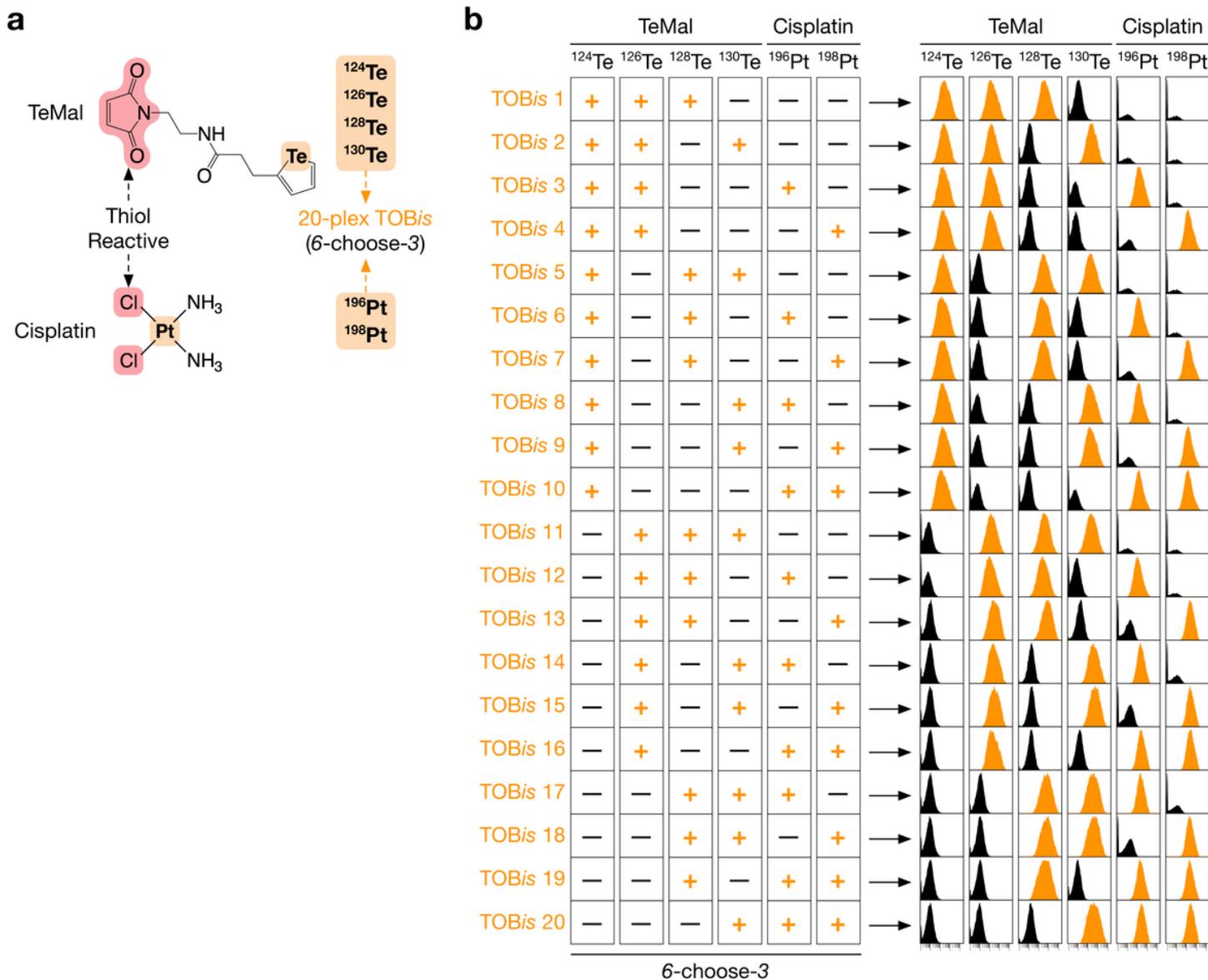


Figure 2

Thiol-reactive Organoid Barcoding in situ (TOBis). a) Thiol-reactive tellurium maleimide (TeMal) and Cisplatin isotopologs combined to form a 20-plex (6-choose-3) doublet-filtering organoid barcoding strategy. b) Small intestinal organoids stained in situ with TOBis 1–20 barcodes according to the 6-choose-3 matrix are successfully resolved into each individual sample.