Multiplexed Single-Cell Analysis of Organoid Signalling Networks

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Editorial Summary This multiplexed mass cytometry protocol uses Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) and a CyTOF siGNalling AnaLysis pipeline (CyGNAL) to enable 126-plex single-cell analysis of cell-type, cell-state, and post-translational modification signalling network in organoids.

Tweet A multiplexed mass cytometry protocol using Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) and a CyTOF siGNalling AnaLysis pipeline (CyGNAL) for 126-plex single-cell analysis of cell-type-specific PTM signalling in organoids (from @QinXiao1990 @FerranC96 and @christophertape).

ABSTRACT

Organoids are biomimetic tissue models comprising multiple cell-types and cell-states. Post-translational modification (PTM) signalling networks control cellular phenotypes and are frequently dysregulated in diseases such as cancer. Although signalling networks vary across cell-types, there are limited techniques to study cell-type-specific PTMs in heterocellular organoids. Here we present a multiplexed mass cytometry (MC) protocol for single-cell analysis of PTM signalling and cell-states in

organoids and organoids co-cultured with fibroblasts and leukocytes. We describe how Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) enables 35-plex and 126-plex single-cell comparison of organoid cultures and provide a CyTOF siGNalling AnaLysis pipeline (CyGNAL) for computing cell-type-specific PTM signalling networks. The TOB*is* MC protocol takes ~3 days from organoid fixation to data acquisition and can generate single-cell data for >40 antibodies from millions of cells across 126 organoid cultures in a single MC run.

INTRODUCTION

Organoids are self-organising biomimetic 3D structures comprising both stem and differentiated cells[1]. Organoids recapitulate many core features of tissue biology and are empowering scientists to study both healthy and diseased tissues *in vitro*[2]. Healthy organoid models of the intestine[3], liver[4], brain[5], and pancreas[6] have been developed, as well as patient-derived organoids (PDOs) as avatars of personalised cancer therapy[7-9]. While incredibly powerful, organoids are heterogenous model systems that are challenging to analyse using conventional technologies.

Cells within an organoid can be classified with a 'cell-type' (e.g., stem, differentiated) and a 'cell-state' (e.g., proliferating, quiescent) – with cell-type often relating to cell-state. For example, stem cells in small intestinal organoids are often in S, G2, and M-phases of the cell cycle, whereas terminally differentiated enterocytes are post-mitotic or apoptotic[10]. Biological processes within all cells are regulated by protein post-translational modification (PTMs) signalling networks[11]. Common PTMs include protein phosphorylation, methylation, acetylation, and ubiquitination[12]. PTM

signalling networks are frequently dysregulated in cancer and PTM signalling nodes such as kinases are targeted by many anti-cancer drugs[13]. As PTM signalling networks are cell-type[14] and cell-state-specific[15], heterocellular organoids contain several cell-type and cell-state-specific PTM networks simultaneously (Fig. 1). Experimental manipulation of organoid cultures such as drug treatments and CRISPR-mediated genome edits can further alter the cell-types, cell-states, and PTM signalling networks in organoids. Unfortunately, low-dimensional technologies commonly applied to organoids cannot measure such high-dimensional changes and therefore fail to capture the complexity of organoid biology[16]. To fully utilise organoids in biomedical research, we must be able to quantify and compare multiple organoid PTM signalling networks in a cell-type and cell-state-specific manner.

We recently described a mass cytometry (MC) (also known as cytometry by time-of-flight (CyTOF)) method to perform cell-type-specific PTM network analysis of organoids and organoid co-cultures[17]. Through the use of heavy-metal tagged probes and antibodies, MC enables >40 protein-level measurements at single-cell resolution across millions of cells[18]. When combined with a Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) strategy, this method enabled cell-type- and cell-state-specific comparison of 28-node PTM networks between 20 different organoid cultures in a single experiment[17]. TOB*is* MC revealed an intimate relationship between cell-type and cell-state PTM signalling in small intestinal organoids and uncovered a novel connection between oncogenic and microenvironmental signalling cues in colorectal cancer (CRC) tumour microenvironment organoid co-cultures[17].

Overview of the Procedure

Here we provide a detailed step-by-step protocol to perform TOB*is* MC analysis of organoids and organoid co-cultures (Fig. 2). The TOB*is* MC protocol comprises four stages: (I) organoid culture and pre-treatment (Steps 1—7), (II) organoid *in situ* barcoding with TOB*is* (Steps 8—10), (III) organoid single-cell dissociation followed by metal-antibody staining and MC data acquisition (Steps 11—49), and (IV) demultiplexing of TOB*is*-barcoded experimental conditions and downstream data analysis (Steps 50—70). The protocol was originally established to study cell-type-specific PTM signalling in murine heterocellular organoids using a 20-plex TOB*is* barcoding strategy (via a *6*-choose-*3* combination, i.e. 3 isotopes 'on', 3 isotopes 'off')[17]. We have now adapted the method to work with smaller 96-well plate organoid cultures, expanded TOB*is* MC to higher-throughput 35-plex (*7*-choose-*3*) and 126-plex (*9*-choose-*4*) formats, demonstrated the protocol is compatible with human PDOs, and developed CyGNAL (CyTOF siGNalling AnaLysis), a computational pipeline for analysing high-dimensional PTM signalling MC data.

Comparison with Other Methods

Immunofluorescent (IF) technologies are limited by the spectral overlap of reporters, susceptible to autofluorescent artefacts, and are inherently low-dimensional. IF imaging parameters can be expanded through multiple cycles of staining and quenching[19], but this is challenging to implement on heterogenous and delicate 3D organoid cultures. While fluorescent flow cytometry (FC) parameters can be increased using compensation strategies, these workflows are complex and not well-suited to the dozens of intracellular measurements required for routine multiplexed

PTM network analysis[20]. In contrast to fluorescent technologies, the Dalton-level mass resolution of MC instruments[21] enables >40 extracellular, intracellular, and nuclear monoisotopic heavy-metal channels to be measured at the single-cell level[18].

High-throughput drug and CRISPR organoid screens rely on bulk viability measurements such as CellTitre-Glo®[7, 9]. Such assays cannot provide cell-type-specific readouts from co-cultures, lack detailed cell-state profiling, and provide no mechanistic insight into organoid phenotypes. Moreover, bulk -omics and low-dimensional fluorescent technologies also struggle to provide multiplexed cell-type-specific PTM signalling data from organoid co-cultures. In contrast, TOB*is* MC provides cell-type-specific cell-state quantification and PTM signalling networks for every cell in an organoid culture. The high-parameter capacity of MC is particularly suited to analysing PTM signalling in organoids co-cultured with stromal fibroblasts and leukocytes[17].

High-dimensional phenotyping of heterocellular systems is commonly performed using single-cell RNA-seq (scRNA-seq)[22, 23]. While a mature technology to identify transcriptionally regulated differentiation trajectories, scRNA-seq workflows to measure the intracellular proteins, PTMs, and biochemical processes[24] are in their infancy. New scRNA-seq methods using intracellular oligo-tagged antibodies have been reported[25, 26], but such methods have not yet been applied to organoids. In comparison, TOB*is* MC is explicitly designed for highly multiplexed protein and PTM measurements. Moreover, as viable cells are needed to achieve suitable read depth and reliable data interpretation for scRNA-seq, dead cells are usually removed during

scRNA-seq sample preparation and stressed cells are excluded in data analysis. Common scRNA-seq workflows are therefore heavily biased towards healthy cells and not well suited to analysing cell death in organoids (as might be common in a PDO drug screen[7]). By contrast, MC can analyse both viable, stressed, and dead cells and is therefore capable of assessing apoptotic mechanisms in perturbed organoid cultures.

Cell-states and PTM signalling are dynamic processes that are rapidly altered by single-cell dissociation[27, 28]. Therefore, organoids should be fixed before dissociation to accurately preserve cell-states and labile PTM signals for molecular analysis. Unfortunately, most scRNA-seq methods are incompatible with PFA fixed cells[23]. In contrast, MC is fully compatible with PFA fixation and can accurately measure cell-states and PTMs from PFA-fixed organoids. Although droplet-based scRNA-seq methods have greatly improved cell-throughput[29, 30], leading commercial platforms (such as 10x Genomics) are commonly limited to ~1-10x10^3 cells per run. Given that a typical 12-well plate organoid culture contains ~0.5-1x10^6 cells per well, droplet-based scRNA-seq methods can only capture ~0.1-1% of the cells in such an experiment. In comparison, TOB*is* MC routinely analyses >1x10^6 single cells and can therefore provide a more holistic view of organoid cultures. Once the protocol is established, data generation is also very rapid. We typically go from fixed organoid cultures to single-cell PTM data in ~3-4 days. Finally, by barcoding organoids very early in the protocol, TOB*is* reduces technical variation between samples in downstream steps (e.g., antibody staining) and increases single-cell recovery[17].

Limitations

As TOB*is* MC requires organoids to be dissociated into a single-cell suspension, all spatial information is lost. Methods such as imaging mass cytometry (IMC)[31], multiplexed ion beam imaging (MIBI)[31], or *in situ* scRNA-seq[32] should be considered when high-dimensional spatial phenotyping is required. While MC can in theory be used to measure any cell sample that can be dissociated into single cells, we have not optimised TOB*is* for tissue samples. We recommend methods specifically optimised for fixed tissue such as DISSECT[28] for measuring PTM signalling in tissue. MC cannot describe intercellular signalling mediated by the thousands of ligand-receptor interactions responsible for transducing signals between cells. We suggest using scRNA-seq ligand-receptor analysis such as CellPhoneDB[33, 34], NicheNet[35] or CellChat[36] to study intercellular communication in organoid co-cultures.

Although we have expanded the capacity of TOB*is* multiplexing to up to 126 different organoid cultures, this is still far below the thousands of conditions assessed in high-throughput screening applications[7]. We therefore still recommend bulk viability measurements when mono-culture assay throughput is paramount.

Like all immunostaining methods, MC is heavily dependent on high-quality antibody reagents. While many PTM and cell-state antibodies are well validated for MC, organoid cell-type identification antibodies are typically less established. Users are advised to screen and validate cell-type identification antibodies when applying this protocol to novel organoid cultures. Like most MC experiments, the cost of TOB*is*

MC is dominated (75-80%) by the price of metal-conjugated antibodies (Supplementary Table 1).

Applications

We have successfully applied TOB*is* multiplexing to 6-well, 12-well, 48-well, and 96-well organoid culture formats to study cell-type-specific PTM signalling networks in organoids and organoids co-cultured with stromal and immune cells[17]. The protocol is also well suited to studying how organoid signalling networks can be regulated by stromal and immune cells, including cellular therapies such as chimeric antigen receptor (CAR) T cells (data not shown). We have successfully applied TOB*is* MC to multiple genotypic and microenvironmental conditions[17]. Although the workflow was developed using murine intestinal organoids, the protocol is compatible with human PDO drug and perturbation screens[7] where mechanisms of cell death and insight into drug resistance are of interest.

EXPERIMENTAL DESIGN

Organoid Culture

This protocol is designed to measure cell-type-specific PTM signalling networks in organoids cultured in a protein-rich extracellular matrix such as Matrigel. Organoids can be grown in 6-well (x7 40 µL droplets), 12-well (x3 30 µL droplets), 48-well (x1 30 µL droplet), and 96-well (x1 50 µL stack) culture formats. As MC can measure both viable and apoptotic cells, the method can be used to analyse both newly seeded and fully developed organoids[17]. The current protocol is optimised for intestinal organoids derived from mouse (Fig. 3) and human (Fig. 4) stem cells cultured in

conventional organoid media[3], but it is theoretically applicable to all Matrigel-based organoid models (e.g., the liver[4], pancreas[6], lung[37], stomach[38], uterus[39], and various cancers[40]) and organoid co-cultures (Fig. 5). Given the highly multiplexed nature of TOB*is* MC, careful consideration should be given to experimental design at the organoid culture stage. We advise users to culture each condition in technical triplicate and to include baseline untreated controls to aid downstream data analysis (see Step 66).

Organoid Pre-Fixation Treatment

Following organoid culture, ^{127}5-Iodo-2'-deoxyuridine (^{127}IdU) is added to the media of live organoid cultures 30 mins prior to assay end-point. ^{127}IdU integrates into the replicating genome of cells in S-phase and can be easily monitored by MC[41]. 5 mins prior to endpoint, a cocktail of protease and phosphatase inhibitors can be added to the organoid culture media to protect protein and phosphorylation epitopes respectively (Supplementary Fig. 1). We advise users to optimise the use of any such pre-treatments with their own biological system and antibody panels.

Organoid Fixation and Dead-Cell Staining

Dissociation of live tissue can alter cell-states[27] and PTM signalling profiles[28]. To avoid disruption of *in situ* cell-states and PTMs in organoids, organoids are fixed while still in Matrigel. At the assay endpoint, culture media is removed and replaced with 4% paraformaldehyde (PFA) and incubated at 37 ºC for 60 minutes. Fixed organoids are then washed with PBS and stained with monoisotopic Cisplatin (e.g., ^{194}Cisplatin or ^{198}Cisplatin) for 10-15 minutes (Supplementary Fig. 2). Cisplatin enters cells with

compromised membranes faster than cells with intact membranes and can therefore be used to identify dead and dying cells in organoid cultures[42].

Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) Multiplexing

Mass-tagged Cellular Barcoding (MCB) is used in MC experiments to increase sample throughput, reduce technical variation, and decrease rare-earth metal conjugated antibody usage[43, 44]. Unfortunately, commercial palladium MCB reagents (commonly used for barcoding leukocytes in suspension) are not suitable for labelling organoids while embedded in Matrigel[17]. To overcome this, we developed Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) based on monoisotopic tellurium maleimide (TeMal)[45] and Cisplatin[46] that can label organoids while still in Matrigel[17] (Fig. 3a-b). We originally reported a 20-plex doublet-filtering barcoding matrix based on ^{124}Te, ^{126}Te, ^{128}Te, ^{130}Te, ^{196}Pt, and ^{198}Pt (using a *6*-choose-*3* combination strategy, i.e. 3 isotopes 'on', 3 isotopes 'off')[17]. Through the addition of ^{122}Te, ^{123}Te, and ^{125}Te TeMals, we have now expanded TOB*is* to support 35-plex (*7*-choose-*3*) (Fig. 3c) or 126-plex (*9*-choose-*4*) multiplexing (Fig. 3d).

TeMals and Cisplatins are pre-mixed into TOB*is* barcodes either by hand or using an Opentrons OT-2 robot (https://github.com/TAPE-Lab/OT-2-Automated-Barcode-Pipetting) and stored as ready-to-use kits. We typically use 35-plex TOB*is* for day-to-day experiments and reserve 126-plex barcoding for screening applications. On the day of the experiment, the TOB*is* barcodes are added to organoid cultures and incubated overnight at 4 ºC. The following day, unbound thiol-reactive barcodes are quenched using reduced glutathione (GSH) and washed from the cultures (Fig. 3b).

These quenching and washing steps avoid unbound barcodes cross-reacting with off-target organoid cells when all cultures are subsequently pooled [17]. When used as described in this protocol, TOB*is* achieved a barcoding accuracy of >99% (Fig. 4) and can be used to stain up to 2 million cells per well of a 96-well plate culture (Supplementary Fig. 3). TOB*is* can be used to multiplex mouse or human organoids and organoids co-cultured with other cell types in 3D, such as stromal fibroblasts and leukocytes (Fig. 5).

Organoid Single-Cell Dissociation and Rare-Earth Metal Conjugated Antibody Staining

Following TOB*is* staining, each organoid culture is removed from Matrigel and resuspended in a dissociation buffer containing Dispase II, Collagenase IV, and DNase I. During optimisation, we found that Dispase II breaks cell-cell contacts, Collagenase IV digests Matrigel ECM components, and DNase I degrades genomic DNA released from dead cells commonly found in organoid cultures. Dissociation enzymes affect cell recovery and antibody staining and should be optimised carefully for each biological system and antibody panel (Supplementary Fig. 4). TOB*is* barcoded organoids from each condition are pooled into a single master tube and then dissociated into single cells using a gentleMACS Octo Dissociator. Following dissociation, single organoid cells are washed and filtered to remove clumps. Cells are then stained for extracellular epitopes with rare-earth metal labelled antibodies (hereafter referred to as metal-antibodies). Metal-antibodies can be purchased via Fluidigm or custom conjugated using established protocols[47]. Cells are then permeabilised using either 0.1% triton X-100, 50% methanol, or both.

Permeabilisation buffers can greatly alter antibody staining and should be optimised for each biological system and antibody panel (Supplementary Fig. 5). Once permeabilised, cells are stained with a panel of metal-antibodies against intracellular-proteins and PTMs. Cells are then washed, and antibodies are cross-linked to their epitopes using 1.6% formaldehyde (FA). Finally, cells are incubated in DNA intercalator $^{191/193}$Ir overnight prior to MC single-cell data acquisition and analysis.

MC Single-Cell Data Acquisition

Stained organoid cells are washed into water containing 2 mM EDTA, diluted to 0.8-1.2x10^6 cells / mL, and spiked with isotopic EQ beads[48]. Cells are then loaded into a Super Sampler (Victorian Airships) and single-cell data is acquired using a mass cytometer (e.g., Fluidigm Helios). During optimisation, we found adding 2 mM EDTA to the running buffer and using the Super Sampler greatly improve the acquisition of epithelial organoid cells by MC (unpublished observation by JS and XQ).

TOB*is* MC Single-Cell Data Analysis

Following data acquisition, raw MC data is normalised[48] and exported as standard FCS file(s). Multiplexed TOB*is* experiments are debarcoded[44] (https://github.com/zunderlab/single-cell-debarcoder) into individual conditions (Fig. 6a), imported into Cytobank (http://www.cytobank.org/) or an equivalent cytometry data analysis platform (e.g. FlowJo), and gated with Gaussian parameters to remove debris, DNA / Cisplatin to identify live cells, and cell-type markers to remove doublets (Fig. 6b). The fully gated datasets containing cells of interest are further processed with our MC data analysis pipeline, CyGNAL (CyTOF siGNalling AnaLysis,

https://github.com/TAPE-Lab/CyGNAL)[49] (Supplementary Fig. 6). The components of CyGNAL were previously used to analyse the datasets described in Qin *et al.*[17]. In brief, the pre-processing step formats and exports the heavy-metal channels (based on the naming convention of the Fluidigm CyTOF Software), embeds the metadata of the experiment, and assigns each event within the dataset a unique cell index. Dimensionality reduction (e.g. UMAP[50]) can be performed on cell-comprised datasets and is mainly used as a visualisation tool in our workflow (Fig. 6c). Cells can be assigned a cell-type identity via biaxial gating (Fig. 6d), followed by cell-state identification and PTM analysis in a cell-type-specific manner (Fig. 6e-f). Earth Mover's Distance (EMD)[51, 52] is used to quantify PTM node intensity, and Density Resampled Estimation of Mutual Information (DREMI)[53] is used to score PTM-PTM edge connectivity. Multiple EMD / DREMI values can be visualised with heatmaps, and further summarised using principal component analysis (PCA). When paired with a well-curated antibody panel and robust experimental design, TOB*is* MC allows multiplexed analysis of cell-type-specific PTM signalling of heterocellular organoids[17].

MATERIALS

Biological Materials

Wild-type, Apc knockdown (*shApc*), and *shApc* / *Kras*$^{G12D/+}$ murine colon organoids[54] (gift from Dr. L. Dow, Cornell University)

Wild-type murine small intestinal organoids (gift from Dr. V. Li, Crick Institute)

Colorectal cancer (CRC) PDOs[7] (gift from Dr. M. Garnett, Sanger Institute)

Immortalised wild-type colonic fibroblasts[17]

Bone marrow derived macrophages[17]

Reagents

Organoid Culture

Growth Factor Reduced Matrigel (Corning, Cat# 354230)

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

L-Glutamine (Thermo, Cat# 25030081)

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

HEPES (Sigma, Cat# H3375)

B-27 Supplement (Thermo, Cat# 17504044)

N-2 Supplement (Thermo, Cat# 17502048)

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

Murine EGF (Thermo, Cat# PMG8041)

Murine Noggin (Peprotech, Cat# 250-38)

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

Murine Wnt-3a (Peprotech, Cat# 315-20)

Human R-spondin-1 (Peprotech, Cat# 120-38)

Gastrin I (Sigma, Cat# SCP0152)

A83-01 (Generon, Cat# 04-0014)

SB202190 (Cayman Chemical, Cat# 10010399)

Nicotinamide (Merck, Cat# N0636)

Mass Cytometry

^{127}5-Iodo-2'-deoxyuridine (^{127}IdU) (Fluidigm, Cat# 201127)

Protease Inhibitor Cocktail (Sigma, Cat# P8340)

PhosSTOP™ (Sigma, Cat# 4906845001)

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

▲CAUTION: PFA is a mutagenic and carcinogenic agent. Avoid eye or skin contact.

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

^{196}Cisplatin (custom order from BuyIsotope)

^{198}Cisplatin (Fluidigm, Cat# 201198)

▲CAUTION: Cisplatin is mutagenic and carcinogenic. Avoid eye or skin contact.

L-Glutathione (Sigma, Cat# G6529)

Dispase II (Thermo, Cat# 17105041)

Collagenase IV (Thermo, Cat# 17104019)

DNase I (Sigma, Cat# DN25)

TeMal (^{122}Te, ^{123}Te, ^{124}Te, ^{125}Te, ^{126}Te, ^{128}Te, ^{130}Te) (see Supplementary Method)

Maxpar® Cell Staining Buffer (Fluidigm, Cat# 201068)

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

Maxpar® Water (Fluidigm, Cat# 201069)

Maxpar® PBS (Fluidigm, Cat# 201058)

Maxpar® Fix and Perm Buffer (Fluidigm, Cat# 201067)

EDTA (Sigma, Cat# 03690-100ML)

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)

Reagent Setup

Organoid Culture Media

Murine colonic organoid monoculture and co-cultures are maintained in advanced DMEM/F-12 supplemented with 2 mM L-Glutamine, 1 mM *N*-acetyl-L-cysteine, 10 mM HEPES, 1× B-27 Supplement, 1× N-2 Supplement, 100 ng/mL murine WNT-3a, 50 ng/mL mEGF, 50 ng/mL mNoggin, 500 ng/mL mR-Spondin-1, 10 mM nicotinamide, and 1× HyClone Penicillin Streptomycin Solution. CRC PDOs are cultured in advanced DMEM/F-12 supplemented with 2 mM L-Glutamine, 1 mM *N*-acetyl-L-cysteine, 10 mM HEPES, 1× B-27 Supplement, 1× N-2 Supplement, 100 ng/mL murine Wnt-3a, 50 ng/mL mEGF, 100 ng/mL mNoggin, 500 ng/mL human R-spondin-1, 10 nM Gastrin I, 500 nM A83-01, 10 µM SB202190, 10 mM nicotinamide, and 1× HyClone Penicillin Streptomycin Solution. TOB*is* MC is theoretically applicable to all Matrigel-based organoid models (see EXPERIMENTAL DESIGN), and users should alter the composition of the organoid culture media based on their model system.

Heavy Metal Conjugated Antibodies

Metal-antibodies can be purchased pre-conjugated from Fluidigm or custom conjugated with monoisotopic heavy metals purchased from Fluidigm or Trace Sciences using X8 polymers as per established protocols[47]. We advise users to develop custom metal-conjugated antibody panels specifically for their biological questions and titrate their panels with prior knowledge such as antigen abundance and heavy metal monoisotopic impurities[55, 56]. In our experience, cell-state (e.g. proliferating, quiescent, and apoptosis) has a considerable influence on PTM signalling[17]. We therefore strongly advise users to include cell-cycle (e.g., pRB [S807/S811], Cyclin B1, Geminin, PLK1, and pHistone H3 [S28])[15, 41, 57] and apoptosis (e.g., cCaspase3 [D175], cPARP [D214]) markers in their panels. Particular care should be taken to validate cell-type identification antibodies that have not previously been used in MC. Ideally >2 cell-type identification antibodies should be used per cell-type. An example metal antibody panel for studying murine small intestinal organoid cells is provided in Supplementary Table 2.

Thiol-reactive Organoid Barcoding *in situ* (TOB*is*)

Debarcoding efficiency is heavily dependent on robust signal intensities of the Te and Pt channels. As barcode signal intensities can vary between isotopologues, barcode batch, and cell-types being labelled, we advise that TeMal and Cisplatin barcodes are titrated to achieve an 'on' median intensity $>5x10^2$ and 'off' median intensity $<1x10^2$ in desired cell-types (Fig. 3c-d). We use TeMals at ^{122}Te = 2.2 µM, ^{123}Te = 2.0 µM, ^{124}Te = 1.8 µM, ^{125}Te = 1.5 µM, ^{126}Te = 1.5 µM, ^{128}Te = 1.1 µM, ^{130}Te = 0.96 µM and both Cisplatins at ^{196}Pt and ^{198}Pt = 125 nM (diluted in PBS). These concentrations

offset the differential mass-range sensitivity of MC instruments and the alternative thiol-reactive functional groups of TeMal (maleimide) and Cisplatin (chloride).

In practice, we prepare TOB*is* barcodes either by hand or robot (see below) and aliquot them as ready-to-use kits (stored in 96-well PCR plates). TeMals and Cisplatins can be mixed at desired concentrations according to the barcoding matrix (Supplementary Tables 3 and 4) manually or using a liquid handling robot. Scripts to prepare both 35-plex (*7*-choose-*3*) and 126-plex (*9*-choose-*4*) TOB*is* barcodes using the OT-2 platform (Opentrons) are provided at: https://github.com/TAPE-Lab/OT-2-Automated-Barcode-Pipetting. Pre-mixed TOB*is* barcodes can be kept at -80 ºC for long-term storage before use. While this protocol is designed for TeMal and Cisplatin reagents, alternative thiol-reactive heavy-metal probes (e.g., lanthanide-conjugated mDOTA[17]) could also, in theory, be used to perform TOB*is*.

EQUIPMENT

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

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

CyTOF Super Sampler (Victorian Airships)

Helios Mass Cytometer (Fluidigm)

Invitrogen™ Countess II Automated Cell Counter (Thermo Fisher)

Equipment Setup

gentleMACS Octo Dissociator Custom Programs

For organoid single-cell dissociation, the following custom programs were designed for the gentleMACS Octo Dissociator:

- Standard Protocol:
    - Set Heater temperature: 37 ºC
    - Forward spin at 20 rpm for 2 min
    - Backward spin at 20 rpm for 2 min
    - Loop 15×:
        - Forward spin at 1500 rpm for 2 sec
        - Backward spin at 1500 rpm for 2 sec
        - Forward spin at 50 rpm for 3 min
- Quick Protocol:
    - Set Heater temperature: 37 ºC
    - Forward spin at 50 rpm for 1 min
    - Backward spin at 50 rpm for 1 min
    - Loop 10×:
        - Forward spin at 1500 rpm for 2 sec
        - Backward spin at 1500 rpm for 2 sec
        - Forward spin at 100 rpm for 1 min

Helios Mass Cytometer

The Helios Mass Cytometer is maintained by procedures recommended by Fluidigm and tuned on each day of MC experiments. The criteria for successful tuning are as follows:

- Resolution (Mass1) is >400.

- The Mean Duals for ^{159}Tb is >600,000 (aim for >1,000,000 if possible).
- The Dual Slopes are between 0.03 and ±0.003.
- The R2 is >0.8.
- If Gas/Current optimization was selected this Oxide ratio (M1/M2) is displayed in Gases, this should be lower than <0.03.
- The %RSD (relative standard deviation) values for Cs, La, Tb, Tm, and Ir should be <3%.

For single-cell data acquisition, the Helios Mass Cytometer is operated at the "Event" mode, with a flow rate of 30 µL / min. We recommend using the 'Wide Bore Injector' when possible to avoid sample blockage.

Software

Fluidigm CyTOF Software (Version 6.7) (https://www.fluidigm.com/software)

Enterprise Cytobank (Version 7.2.0) (https://cytobank.org)

Graphpad Prism (Version 7.0) (https://www.graphpad.com)

CyGNAL (Version 0.2.1)[49] and its dependencies (https://github.com/TAPE-Lab/CyGNAL/releases/tag/v0.2.1)

- Python >3.6 (https://www.python.org/) with libraries:
  - *fcsparser*
  - *fcswrite*
  - *numpy*
  - *pandas*
  - *plotly*

480 - *rpy2*
481 - *scprep*
482 - *sklearn*
483 - *umap-learn*
484 • R >3.6 (https://www.r-project.org/) with libraries:
485 - *ComplexHeatmap*
486 - *DT*
487 - *factoextra*
488 - *FactoMineR*
489 - *flowCore*
490 - *Ggally*
491 - *ggrepel*
492 - *ggplot2*
493 - *Hmisc*
494 - *MASS*
495 - *matrixStats*
496 - *plotly*
497 - *psych*
498 - *RColorBrewer*
499 - *shiny*
500 - *tidyverse*

501 MATLAB (https://www.mathworks.com/products/matlab.html):

502 • Single Cell Debarcoder (https://github.com/zunderlab/single-cell-
503 debarcoder)

PROCEDURE

TOB*is* MC can be used to assess organoids cultured in 6-well, 12-well, 48-well, or 96-well formats. This protocol describes how to analyse intestinal organoids grown either in monoculture or co-cultured with intestinal fibroblasts and/or primary bone marrow derived macrophages in a 96-well plate. Users are advised to deploy their own optimised organoid culture conditions as inputs for TOB*is* MC.

Culture Organoids

1) Culture organoids (or organoid co-cultures) in 50 µL Matrigel and 200 µL media in a standard 96-well tissue culture plate.

▲ **CRITICAL STEP:** For the data in this paper, we have cultured murine intestinal organoids (Figure 3), human CRC PDOs (Figure 4), and murine organoids co-cultured with colonic fibroblasts and/or macrophages (Figure 5) for 3 days in 50 µL Matrigel and 200 µL media. Users should use optimised culture conditions relevant to their own organoids and biological questions as inputs for TOB*is* MC.

S-Phase Cell Labelling ◉ TIMING: ~30 min

2) Add ^{127}IdU directly to culture media to a final concentration of 25 µM (10 µL of 0.5 mM stock added to 200 µL media). Gently rotate the plate by hand 5 times for 10 seconds to mix media and incubate the plate for 25 min at 37 ºC, 5 % CO_2.

▲ **CRITICAL STEP:** ^{127}IdU incubation enables identification of S-phase cells.

Phosphatase & Protease Inhibitor Treatment ◉ **TIMING:** ~5 min

3) Add the protease inhibitor cocktail (100× stock, see REAGENTS) and PhosSTOP (40× stock, see REAGENTS) directly to culture media, gently rotate the plate by hand 5 times for 10 seconds and incubate for 5 min at 37 ºC, 5 % CO_2.

▲ **CRITICAL STEP:** Protease and phosphatase inhibitors have been shown to help preserve cell signalling and antigen stability during fixation[28]. However, as prolonged treatment may introduce technical artefacts, we advise users to empirically determine the duration of the treatment according to their experimental system and antibody panel (Supplementary Fig. 1).

Fixation ◉ **TIMING:** ~80 min

4) Remove culture media by pipetting. Add 200 µL pre-warmed (37 ºC) 4% PFA into each well taking care not to disrupt Matrigel. Incubate for 60 min at 37 ºC, 5 % CO_2.

▲ **CRITICAL STEP:** PFA fixation *in situ* ensures that labile cell-state and PTM profiles are preserved during the downstream sample handling.

▲ **CRITICAL STEP:** Matrigel can dissolve in cold PFA. It is therefore important to pre-warm PFA to 37 ºC.

▲ **CRITICAL STEP:** Some antibodies are sensitive to fixation. PFA concentrations ranging from 1.6% to 4% were proved to be functional, but we encourage users to determine the optimal concentration of PFA for their specific antibody panel.

? TROUBLESHOOTING

5) Remove PFA solution by pipetting, taking care not to disturb the Matrigel. Wash the cells with PBS on a rocker (speed set at ~45 rpm throughout the protocol) for 10 min at room temperature (~20 ºC). Repeat wash.

◆ **PAUSE POINT:** Fixed cells can be kept at 4 ºC in PBS. We advise users to determine the maximal storage time with their specific culture systems.

Live / Dead Discrimination ◉ **TIMING:** ~30 min

6) Remove PBS by pipetting. Add 200 µL of 0.25 µM ^{194}Cisplatin / PBS solution to each well and incubate for 10-15 min on a rocker at room temperature.

▲ **CRITICAL STEP:** As dead cells can be found inside organoid structures (Fig. 1), it is crucial that organoids are stained for long enough that all cells have the opportunity to bind Cisplatin. However, organoids can also be easily overstained with Cisplatin, it is therefore important that cultures are stained for the same duration of time and this step does not exceed 20 min (Supplementary Fig. 2).

7) Remove the ^{194}Cisplatin solution by pipetting. Wash cells with PBS on a rocker for 10 min at room temperature. Repeat wash.

▲ **CRITICAL STEP:** Proceed to the next steps on the same day. Long-term storage of Cisplatin-stained cells *in situ* will lead to Cisplatin overstain that confounds live / dead cell discrimination.

▲ **CRITICAL STEP:** If barcoding multiple organoid samples, continue to Step 8. If only one organoid culture condition is being analysed, skip to Step 11.

Thiol-reactive Organoid Barcoding *in situ* (TOB*is*) (Optional) ◉ **TIMING:** ~30 min bench work; incubation overnight

8) Transfer 200 µL pre-aliquoted TOB*is* barcodes to corresponding organoid samples in a 96-well plate (From Step 7). Any barcode combination can be used to stain any culture condition (Supplementary Tables 3 and 4). Incubate the cells overnight at 4 ºC.

▲ **CRITICAL STEP:** Record the sample-barcode assignments. Different samples labelled with the same TOB*is* barcode should not be pooled together.

▲ **CRITICAL STEP:** Ensure correct amounts of TOB*is* barcode are added to each well for successful debarcoding.

▲ **CRITICAL STEP:** TOB*is* barcodes should not be used to stain >1 million cells per well of a 96-well culture (Supplementary Fig. 3). In practice, culturing > 1 million cells per well of a 96-well plate is uncommon. The users are advised to count cells at seeding if highly dense cultures are needed.

9) Remove the barcoding solutions by pipetting and wash the cells with 200 µL of 2 mM Glutathione / CSB for 10 mins on a rocker at room temperature. Repeat wash twice.

▲ **CRITICAL STEP:** Reduced Glutathione quenches unused thiol-reactive TOB*is* barcodes, thereby enabling efficient discrimination of 'on' and 'off' signals for sample demultiplexing.

10) Wash the cells with 200 µL of PBS for 10 min on a rocker at room temperature. Repeat wash.

◆ **PAUSE POINT:** The barcoded cells can be kept at 4 ºC for up to four weeks in PBS.

Single-Cell Dissociation ◉ **TIMING:** ~90 min

11) 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 at room temperature.

▲ **CRITICAL STEP:** Dissociation enzymes can affect cell recovery and antibody performance (Supplementary Fig. 4). We encourage users to test and titrate alternative dissociation enzymes for the specific cellular composition of their experimental system.

▲ **CRITICAL STEP:** Using freshly prepared enzyme solutions ensures optimal and reproducible enzyme activity.

12) Remove PBS from the wells by pipetting and add the dissociation solution.

13) Scrape Matrigel droplets and pool all cells from all conditions with dissociation solution to a gentleMACS C-tube. Top up the dissociation solution to 5 mL / C-tube.

▲ **CRITICAL STEP:** Do not overload the gentleMACS C-tubes. We encourage users to empirically determine how much dissociation buffer is needed based on the density of their organoid cultures and the number of conditions. If multiple C-tubes are needed, the user should pool all barcoded cells before splitting them evenly into each C-tube to minimise technical variation.

▲ **CRITICAL STEP:** Fibroblasts and leukocytes can migrate out of the central Matrigel droplet and adhere to the plastic bottom of the culture plates in prolonged co-cultures. Scrape each well thoroughly to ensure all cells are recovered.

14) Dissociate organoids into single cells using a gentleMACS Octo Dissociator using the 'Standard Protocol' (see Equipment Setup) (◉ **TIMING:** ~50 min).

▲ **CRITICAL STEP:** On completion of the program, the user needs to confirm visually that the dissociation is sufficient, i.e., very few cell clumps should be visible at this stage. If not, users are encouraged to perform an additional round of the 'Quick Protocol' (see Equipment Setup) on the gentleMACS Octo Dissociator.

? TROUBLESHOOTING

15) After sufficient dissociation, centrifuge the C-tubes at 800×g for 1 min at room temperature to collect the cells.

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

▲ **CRITICAL STEP:** Organoid cells often pellet better in polypropylene than polystyrene FACS tubes.

17) Centrifuge cells at 800×g for 5 min at room temperature and discard supernatant.

18) Wash cells with 2 mL CSB, centrifuge at 800×g for 5 min at room temperature, and discard supernatant. Repeat wash.

19) Resuspend cells in 2 mL CSB and filter through a cell strainer to get rid of residual cell clumps.

▲ **CRITICAL STEP:** We use 35 µm cell strainers to filter organoid monocultures and 70 µm cell strainers for cultures containing large cells such as fibroblasts. Users should choose appropriate cell strainers based on the cellular composition of their experimental system.

20) Count cells using the Countess II Automated Cell Counter. Up to ~4.5×10^6 cells can be taken forward for 1× MC staining.

? TROUBLESHOOTING

◆ **PAUSE POINT:** The fixed, barcoded, and dissociated cells can be kept at 4 ºC for up to four weeks in CSB.

Extracellular Stain ◉ **TIMING:** ~45 min

21) Centrifuge cells at 800×g for 5 min at room temperature, discard supernatant, and resuspend cells in 50 µL CSB.

22) Prepare extracellular antibody cocktail by mixing the antibody panel at desired concentrations in CSB (total volume up to 50 µL).

23) Add the extracellular antibody cocktail to the cells, mix thoroughly by pipetting, and incubate for 30 min on a rocker at room temperature.

▲ **CRITICAL STEP:** Mix cells by gently flicking the tube every 10 minutes to avoid cells pelleting under gravity.

24) Add 2 mL CSB to the cells, centrifuge at 800×g for 5 min at room temperature, and discard supernatant.

Permeabilisation ◉ **TIMING:** ~45 min

25) Resuspend cells in 1 mL 0.1 % Triton X-100 / PBS, gently vortex, and incubate for 30 min on a rocker at room temperature.

▲ **CRITICAL STEP:** Mix cells by gently flicking the tube every 10 minutes to avoid cells pelleting.

26) Add 2 mL CSB to the cells, centrifuge at 800×g for 5 min at room temperature, and discard supernatant. Repeat wash and remove supernatant.

27) Place the cells on ice for 1 min.

28) Resuspend cells in 1 mL ice-cold 50% Methanol / PBS (store at -20 ºC until use), gently vortex, and incubate for 10 min on ice.

▲ **CRITICAL STEP:** Different permeabilisation buffers can substantially alter antibody staining (Supplementary Fig. 5). Although we use commonly use 0.1% Triton X-100 followed by 50% methanol, we advise users to optimise the permeabilisation conditions that best suit their model system and antibody panel.

29) Add 2 mL CSB to the cells, centrifuge at 800×g for 5 min at room temperature, and discard supernatant. Repeat wash.

30) Resuspend cells in 50 µL CSB.

Intracellular Stain ◉ **TIMING:** ~45 min

31) Prepare intracellular antibody cocktail by mixing the antibody panel at desired concentrations in CSB (total volume up to 50 µL).

32) Add the intracellular antibody cocktail to the cells, mix thoroughly by pipetting, and incubate for 30 min on a rocker at room temperature.

▲ **CRITICAL STEP:** Mix the cells by gently flicking the tube every 10 minutes to avoid cells pelleting.

33) Add 2 mL CSB to the cells, centrifuge at 800×g for 5 min at room temperature, and discard supernatant.

Post-Staining Fixation ◉ **TIMING:** ~15 min

34) Add 1 mL 1.6% formaldehyde (FA) / PBS solution made fresh from 16% FA to the cells and incubate for 10 min on a rocker at room temperature.

▲ **CRITICAL STEP:** Post-staining fixation step is required if the sample needs to be stored for more than 48 hours before data acquisition.

35) Add 2 mL CSB to the cells, centrifuge at 800×g for 5 min at room temperature, and discard supernatant.

DNA Intercalation ◉ **TIMING:** ~1 hr / overnight

36) Prepare intercalation buffer by diluting 1 µL 125 µM Cell-ID Intercalator-Ir in 1 mL Fix & Perm Buffer (final concentration = 125 nM).

37) Resuspend cells in the 1 mL of intercalation buffer, gently vortex, and incubate for 1 hour on rocker at room temperature or overnight at 4 ºC.

▲ **CRITICAL STEP:** Since the intercalation reaction is non-covalent, cells should be kept at 4 ºC in the intercalation buffer until ready to proceed to data acquisition (e.g., when the Helios is tuned).

▲ **PAUSE POINT:** Cells can be stored at 4 ºC in the intercalation buffer for up to 2 weeks (with post-staining fixation) or 48 hours (without post-staining fixation).

MC Data Acquisition ◉ **TIMING:** ⩾ 1 hr (dependent on the scale of the experiment)

38) Tune the Helios Mass Cytometer (see 'Equipment Setup').

▲ **CRITICAL STEP:** A reproducible tuning procedure ensures predictable 'on' and 'off' intensities of the TOB*is* barcode channels.

▲ **CRITICAL STEP:** For prolonged MC runs (e.g., when the acquisition lasts for >4 hours), we advise users to perform the 'Quick Tuning Protocol' implemented in the Fluidigm CyTOF Software to ensure consistent signal intensity within the same experiment.

39) Centrifuge cells at 800×g for 5 min at room temperature, and discard supernatant.

40) Wash the cells with 2 mL of 2 mM EDTA / CSB, centrifuge at 800×g for 5 min at room temperature, and discard supernatant.

▲ **CRITICAL STEP:** EDTA chelates free metals in the cell suspension and can clean up MC data acquisition. Do not exceed 2 mM EDTA.

41) Wash cells with 2 mL CSB, centrifuge at 800×g for 5 min at room temperature, and discard supernatant.

42) Wash cells with 2 mL MaxPar Water, centrifuge at 800×g for 5 min at room temperature, and discard supernatant.

43) Resuspend cells in 1 mL of MaxPar Water, filter through a 35 μm cell strainer (70 μm when the culture contains fibroblasts) and count the cells using the Countess II Automated Cell Counter.

44) Dilute cells to ~0.8 – 1.2 × 10^6 / mL in MaxPar Water.

45) Add EQ Beads to the cell suspension at a volumetric ratio of 1:5.

46) Add EDTA to the cells to a final concentration of 2 mM.

▲ **CRITICAL STEP:** EDTA reduces cell clumps during data acquisition.

47) Set up the "Super Sampler" (Victorian Airships) as per the manufacturer's instructions, and set up acquisition parameters (e.g., antibody panel, experiment metadata) on the Helios mass cytometer.

▲ **CRITICAL STEP:** To avoid blockage and ensure smooth data acquisition, we advise users to use the "Super Sampler" to load organoid cells / fibroblasts to the Helios mass cytometer.

48) Acquire events on the Helios mass cytometer using the Fluidigm CyTOF software. Aim for 100-400 events per second.

49) After all events are acquired, process the raw data using the Fluidigm CyTOF software as per Fluidigm recommendation (i.e., signal normalisation, removal of EQ Beads, and concatenation of data files if needed). Export data as FCS file(s) (Fig. 6a, Raw.fcs).

? TROUBLESHOOTING

Debarcoding TOB*is* Multiplexed MC Dataset ◉ **TIMING:** ~15 min

50) Debarcode multiplexed FCS file(s) (Fig. 6a, Raw.fcs) into separate experimental conditions using the MATLAB program Zunder Lab Single Cell Debarcoder (https://github.com/zunderlab/single-cell-debarcoder)[44] with user-defined TOB*is* Barcode Keys (Supplementary Files 1 and 2).

? TROUBLESHOOTING

Installation of CyGNAL ◉ **TIMING:** ~20 min

51) Download the CyGNAL v0.2.1 from the GitHub repository (https://github.com/TAPE-Lab/CyGNAL/releases/tag/v0.2.1) and open a terminal session from the repository folder (/CyGNAL-0.2.1, hereafter referred to as 'pipeline folder').

52) Set up computing environment (see 'Software'). We recommend using Conda (https://docs.conda.io/projects/conda/en/latest/index.html) to recreate the environment defined in conda_env.yml by running the following from the pipeline folder:

```
conda env create -f conda_env.yml
conda activate cygnal
```

▲ **CRITICAL STEP:** All required libraries need to be installed at the recommended versions for successful execution of the pipeline (see 'Software').

? TROUBLESHOOTING

Single-Cell Organoid Data Pre-Processing ◉ **TIMING:** ~45 min

53) Import debarcoded FCS files to the Cytobank platform (http://www.cytobank.org/) or an equivalent FCS processing software (e.g., FlowJo).

54) Perform Gaussian gating to remove debris (Fig. 6b).

55) Perform DNA / Cisplatin gating to identify cells (Fig. 6b).

▲ **CRITICAL STEP:** Different cell-types may display distinct abundances of DNA. Users are advised to check every experimental condition to ensure all cell-types of interest are included in the DNA gating step.

▲ **CRITICAL STEP:** The Cisplatin^{high} population contains both dead and dying cells. If cell death is of biological interest (e.g., in a drug screening assay), we suggest users be more lenient with the Cisplatin gating to include dying cells.

56) Perform cell-type gating to exclude doublets (i.e., cells positive for mutually exclusive cell-type markers) (Fig. 6b).

57) Export the dataset as FCS files and proceed to data analysis with CyGNAL.

▲ **CRITICAL STEP:** The dataset now contains events that are identified as single cells (Fig. 6b, TOBis_n_Cells.fcs).

58) To pre-process dataset(s) using CyGNAL, copy the cell-comprised FCS file(s) to the Raw_Data folder within the pipeline folder, run `python code/1-data_preprocess.py`, and follow the prompts. This step will generate two outputs: the pre-processed datasets and the file panel_markers.csv containing all the markers used in the experiment. The output files are saved in a folder named by the user-defined analysis identifier within the Preprocessed_Data folder (Fig. 6b, Supplementary Fig. 6).

▲ **CRITICAL STEP:** Pre-processing of dataset(s) by 1-data_preprocess.py is prerequisite for the subsequent analysis steps and has to be performed as the first step of the workflow.

▲ **CRITICAL STEP:** panel_markers.csv is used by downstream scripts to specify the markers of interest in a given analysis.

▲ **CRITICAL STEP:** The input file formats supported by CyGNAL are standard FCS and tab-separated ASCII TXT. Users can choose to save the output as either TXT or FCS file(s) (stripped of original FCS metadata), with the default set to match the input file format (example files can be accessed at the GitHub repository).

Dimensionality Reduction (UMAP) ◉ **TIMING:** 20 – 40 min (~1M cells)

59) Copy CyGNAL-processed dataset(s) and the corresponding panel_markers.csv to the Analysis/UMAP_input/ folder and edit panel_markers.csv by labelling markers used for UMAP calculation with 'Y' (by default all markers are labelled with 'N').

60) Run `python code/2-umap.py` from the pipeline folder and follow the prompts. The original dataset updated with UMAP coordinates will be formatted as TXT file(s) and saved in a folder named by the user-defined analysis identifier in Analysis/UMAP_output/ (Fig. 6c, Supplementary Fig. 6).

▲ **CRITICAL STEP:** When performing UMAP analysis on multiple conditions within the same experiment, the 2-umap.py script will concatenate all the input files, calculate UMAP coordinates for the concatenated dataset, and save the results as separate conditions based on their file of origin. This ensures that all the input files share a common UMAP embedding to facilitate direct comparison between conditions.

▲ **CRITICAL STEP:** When multiple files are used for UMAP analysis, users can down-sample all input files to the cell count of the sample with the lowest cell number (details on which cells are included for the analysis can be found in the output folder). This yields a more balanced dataset for UMAP calculation and visualisation and reduces memory requirements and computation time.

Cell-Type and Cell-State Identification ◉ TIMING: ~ 45 min

61) Import CyGNAL-processed dataset(s) to Cytobank or an equivalent FCS processing software (e.g., FlowJo).

62) Identify different cell-types present in the experimental condition(s) based on cell-type-specific markers (Fig. 6d).

▲ **CRITICAL STEP:** To improve the fidelity of cell-type identification, at least 2 markers should be used per cell-type. Ectopically expressed cell-type-specific fluorescent proteins such as GFP and RFP are useful for cell-type identification when robust endogenous antigens are unavailable.

63) For each identified cell-type, perform cell-state analysis based on cell-state markers (Fig. 6e).

64) Export the cell-type and/or cell-state-specific dataset(s) as FCS or TXT files for PTM signalling network analysis.

▲ **CRITICAL STEP:** If using Cytobank, uncheck 'Include header with FCS filename' when exporting TXT files and make sure the dataset(s) is exported as raw values (Cytobank gives users the option to export illustration-based transformed data).

PTM Signalling Network Analysis ◉ **TIMING:** 20 – 40 min (dependent on the scale of the experiment)

65) Copy CyGNAL-processed, cell-type-specific dataset(s) and the corresponding panel_markers.csv to Analysis/EMD_input/ or Analysis/DREMI_input/ and edit panel_markers.csv by labelling markers used for the calculation with 'Y' (by default all markers are labelled with 'N').

66) Run `python code/3-emd.py` or `python code/4-dremi.py` and follow the prompts. The output will be saved in folders named by the user-defined analysis identifier within Analysis/EMD_output/ or Analysis/EMD_output/

accordingly. EMD and DREMI scores can be visualised using heatmaps (Steps 67-68) or summarised with principal component analysis (PCA) (Steps 69-70).

▲ **CRITICAL STEP:** For EMD calculations, the user needs to define the reference (*R*) dataset for all experimental variables (*V*) to compare against. By default, the concatenation of all input files is used as *R*, but users can also assign a specific dataset as the reference.

▲ **CRITICAL STEP:** The choice of *R* can greatly influence the interpretation of EMD scores. When there is a clear baseline control in a given experiment (e.g., untreated monoculture), that control population should be used as *R*. However, when there is no obvious baseline condition (e.g., when comparing PTMs between different cell-types within organoids), we advise using a concatenated population of all conditions as *R* (default setting).

▲ **CRITICAL STEP:** EMD is a non-negative metric quantifying the difference between two distributions. In our workflow, EMD scores are signed by the difference of a marker's median intensity between *V* and *R* in order to indicate the 'direction' of signalling change — positive for up-regulation and negative for down-regulation.

▲ **CRITICAL STEP:** For DREMI calculations, users can perform standard deviation-based outlier removal or generate conditional probability plots for each marker combination. Note: these optional settings increase computational load.

Heatmap Visualisation ◉ **TIMING:** < 5 min

67) To visualise EMD / DREMI scores using heatmaps, copy the output of EMD / DREMI calculations to the Analysis/Vis_Heatmap folder.

68) Open a terminal from the pipeline folder, run `python code/5v1-heatmap.py`, and perform interactive heatmap visualisation in the prompted browser window. Once a satisfactory layout has been achieved, the heatmaps can be exported as a PNG image or PDF document.

PCA ◉ **TIMING:** < 5 min

69) To perform PCA, copy the output of EMD / DREMI calculations to the Analysis/Vis_PCA folder.

70) Run `python code/5v2-pca.py` and perform interactive heatmap visualisation in the prompted browser window. Once a satisfactory layout has been achieved, the heatmaps can be exported as a PNG image or PDF document. The PCA coordinates with the percentage of variance explained by each PC can be exported separately as a TXT file.

TIMING

The pre-treatment and fixation of organoids: around 2 hours.

Live / dead discrimination staining: around 0.5 hour.

TOB*is* barcoding: 1 to 2 hours at room temperature or overnight at 4 ºC.

Quenching of TOB*is* barcodes: around 0.5 hour.

Single-cell dissociation: 2 to 2.5 hours.

MC staining: around 4 hours.

DNA intercalation: 1 hour at room temperature or overnight at 4 ºC.

Data acquisition: 1 to 2 hours depending on the number of cells to be analysed.

Data analysis: 1 to 2 hours depending on the scale of the experiment.

TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

Table 1. Troubleshooting advice.

Step	Problem	Possible Reason	Solution
4	Matrigel droplets dissolve in PFA.	PFA solution not warm enough.	Prewarm 4% PFA solution to 37 ºC prior to the fixation step. Be careful not to disturb the Matrigel droplets when adding PFA to the wells.
14	Organoids are not dissociated properly.	gentleMACS C-Tubes are overloaded, or the dissociation enzymes are performing at suboptimal activity.	In our experience, up to ~ 5×10^6 cells per C-Tube can be dissociated sufficiently using our custom dissociation program. We recommend users to prepare fresh dissociation solution before each use. Run additional rounds of the “Quick Protocol” if needed.
20	Considerable cell loss, especially after being transferred to a new FACS tube.	Cell loss is inevitable during staining due to the multiple washing steps, and it is more striking with fewer cells. In particular, when cells are centrifuged in uncoated polypropylene FACS tubes, a thin film of cells will form on the side of the tube instead of a well-defined cell pellet, leading to further cell loss.	We recommend users start with > $1x10^6$ cells in total, barcode 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 increases cell recovery.

49	Antibody staining is not working.	Antibody needs to be titrated, or alternative antibodies / clones need to be tested.	Antibody panels for MC experiments need to be carefully designed and titrated in accordance with known impurities and antigen abundance[55, 56]. We also encourage users to test alternative fixation and permeabilisation conditions for their specific experimental system.
50	Experimental conditions are not debarcoded efficiently.	a) Incorrect amounts of barcodes are added to the cells. b) The MC run acquires heavy metal contaminants accumulated during the staining steps, causing lower than expected percentage of "real" events. c) Incompatible barcode key is provided to the Debarcoder.	a) Ensure barcodes are accurately aliquoted and all barcodes from each TOB*is* condition is added to the cell cultures. b) Adding 2 mM of EDTA to the CSB wash buffer prior to MC data acquisition helps chelate free metals in the cell suspension and clean up the MC run. c) Make sure the correct barcode key is used for debarcoding a specific experiment.
52	Error messages when installing Conda environment or running pipeline scripts.	Python or R package(s) failed to be installed in the Conda environment at the recommended versions.	a) Identify missing package(s) (see error information of `conda env setup` or run a pipeline script and check which python import fails). Manually install the package(s) as per their specific instructions. b) Errors in R installation are likely caused by missing compilation tools in the operating system (macOS in particular). Identify the missing tools and manually install them. Execution of

			5v1-emd_dremi_htmp.py or 5v2-pca.py should also trigger automatic installation of any missing R packages. c) Refer to the GitHub issue page for additional help.

ANTICIPATED RESULTS

This TOB*is* MC protocol typically generates >1,000,000 single-cell measurements of ~50 MC channels (cell-type identification antibodies, cell-state antibodies / probes, and PTM antibodies) from up to 126 organoid cultures (Fig. 3d). When compared with the starting cell count (i.e., after single-cell dissociation), 50—60% of the cells can be acquired by MC and the sum of TOBis_n_Cells.fcs cell counts typically ranges from 50—70% of the total debarcoded event count. Previous analysis with small intestinal organoids confirmed cell-type and cell-state recovery was in line with expected ratios for small intestinal epithelia[17]. The standard output files generated by the workflow are formatted as FCS 3.0, which is compatible with third party cytometry data analysis tools including Cytobank and FlowJo, where manual data pre-processing, cell-type identification, and cell-state classification can be performed. We do not routinely perform compensation[58] or batch correction[59] on TOB*is* MC data, but such strategies could be useful for some users. Single-cell data can be visualised using UMAP[50] via CyGNAL (or *t*-SNE[60] / PHATE[61] using standalone scripts) and cell-type-specific organoid PTM network analysis is performed with EMD[51, 52] (node) and DREMI[53] (edge) scoring. EMD and DREMI scores output to .csv format and can be easily visualised using heatmaps and PCA. The cell-type-specific results generated by this TOB*is* MC protocol can be used to quantitively compare cell-states and PTM signalling networks between organoids and organoid co-cultures.

930

931 **Reporting Summary**

932 Further information on research design is available in the Nature Research Reporting
933 Summary linked to this article.

934

935 **Data Availability**

936 All raw data, processed data, and working illustrations are available as a Community
937 Cytobank project (https://community.cytobank.org/cytobank/experiments#project-
938 id=1334).

939

940 **Code Availability**

941 The latest CyGNAL pipeline is available at: https://github.com/TAPE-Lab/CyGNAL.
942 CyGNAL version 0.2.1 as described in this publication can be found at:
943 https://github.com/TAPE-Lab/CyGNAL/releases/tag/v0.2.1. The OT-2 barcode
944 preparation code is available at: https://github.com/TAPE-Lab/OT-2-Automated-
945 Barcode-Pipetting.

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AUTHOR CONTRIBUTIONS

J.S. developed TOB*is*, designed rare-earth metal conjugated antibody panels, and performed MC analysis. X.Q. designed and performed organoid and MC experiments, analysed the data, and wrote the paper. F.C.R. developed CyGNAL and wrote the paper. P.V. and M.R. performed organoid and MC experiments. Y.J.B. and M.N. developed TeMal reagents. C.T. designed the study, analysed the data, and wrote the paper.

COMPETING INTERESTS

M.N. has pending intellectual property on the use of TeMal reagents for mass cytometry applications which has been licensed to Fluidigm Corporation.

Figure 1

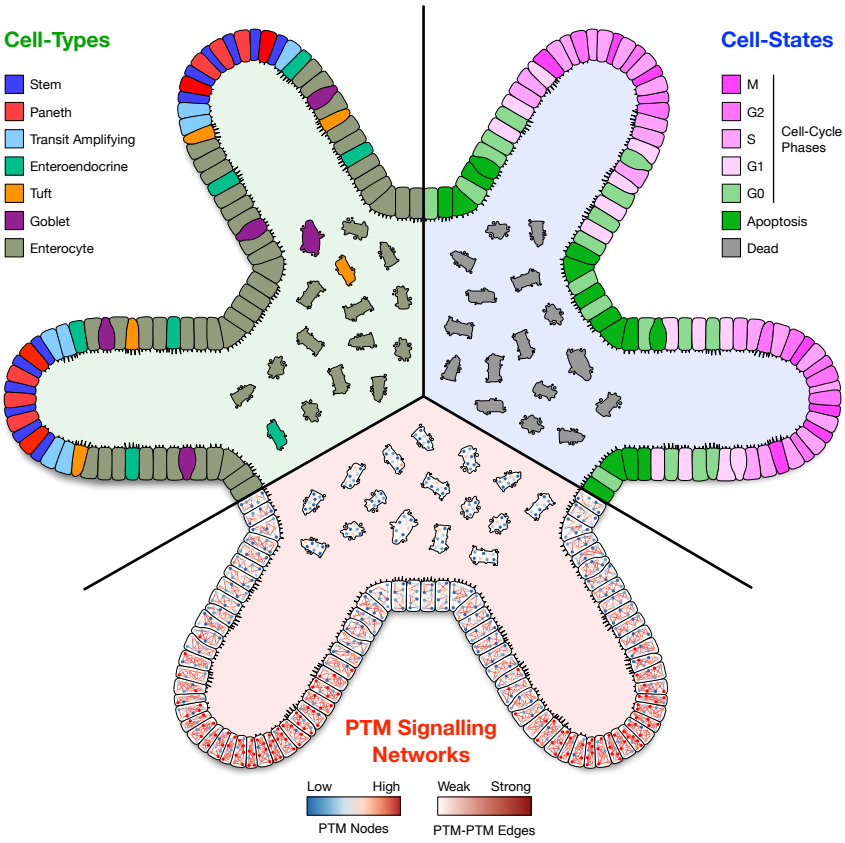

Cell-Types
Stem
Paneth
Transit Amplifying
Enteroendocrine
Tuft
Goblet
Enterocyte
Cell-States
M
G2
S
G1
G0
Cell-Cycle Phases
Apoptosis
Dead
PTM Signalling Networks
Low
High
PTM Nodes
Weak
Strong
PTM-PTM Edges

Figure 2

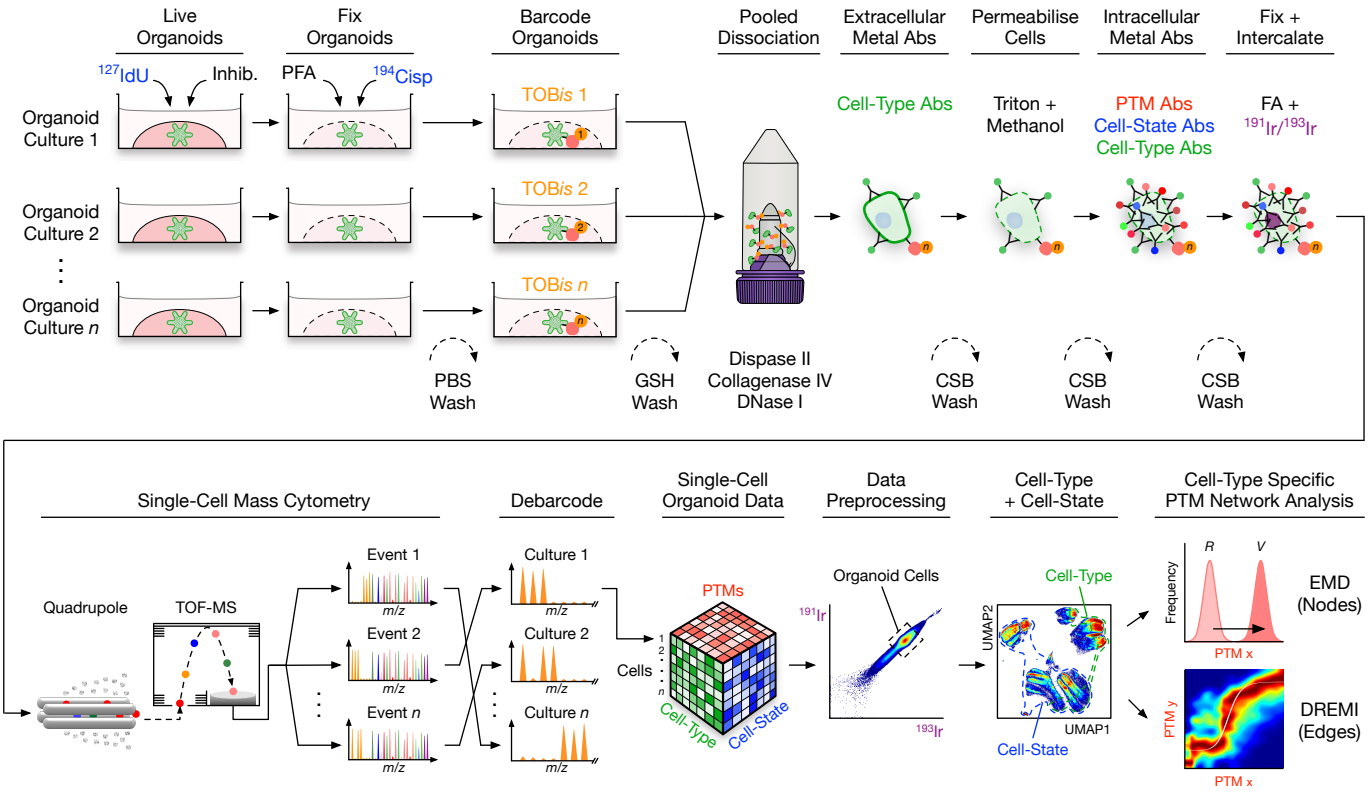

Live Organoids
Fix Organoids
Barcode Organoids
Pooled Dissociation
Extracellular Metal Abs
Permeabilise Cells
Intracellular Metal Abs
Fix + Intercalate
127IdU
Inhib.
PFA
194Cisp
Organoid Culture 1
Organoid Culture 2
Organoid Culture n
TOBis 1
TOBis 2
TOBis n
Cell-Type Abs
Triton + Methanol
PTM Abs
Cell-State Abs
Cell-Type Abs
FA +
191Ir/193Ir
PBS Wash
GSH Wash
Dispase II
Collagenase IV
DNase I
CSB Wash
CSB Wash
CSB Wash
Single-Cell Mass Cytometry
Debarcode
Single-Cell Organoid Data
Data Preprocessing
Cell-Type + Cell-State
Cell-Type Specific PTM Network Analysis
Quadrupole
TOF-MS
Event 1
Event 2
Event n
Culture 1
Culture 2
Culture n
m/z
PTMs
Cells
Cell-Type
Cell-State
Organoid Cells
191Ir
193Ir
UMAP2
UMAP1
Cell-Type
Cell-State
Frequency
R
V
PTM x
PTM y
EMD (Nodes)
DREMI (Edges)

Figure 3

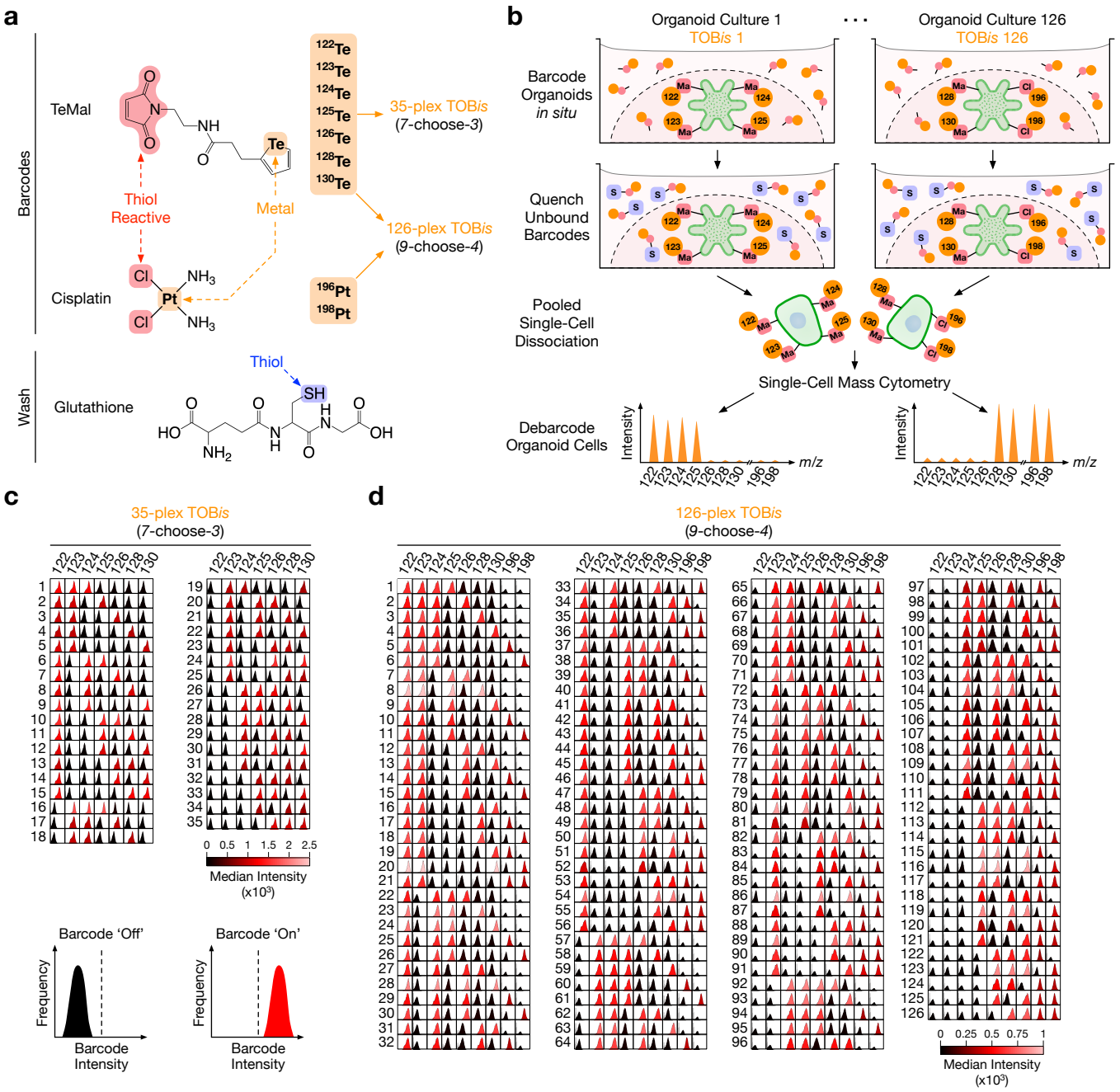
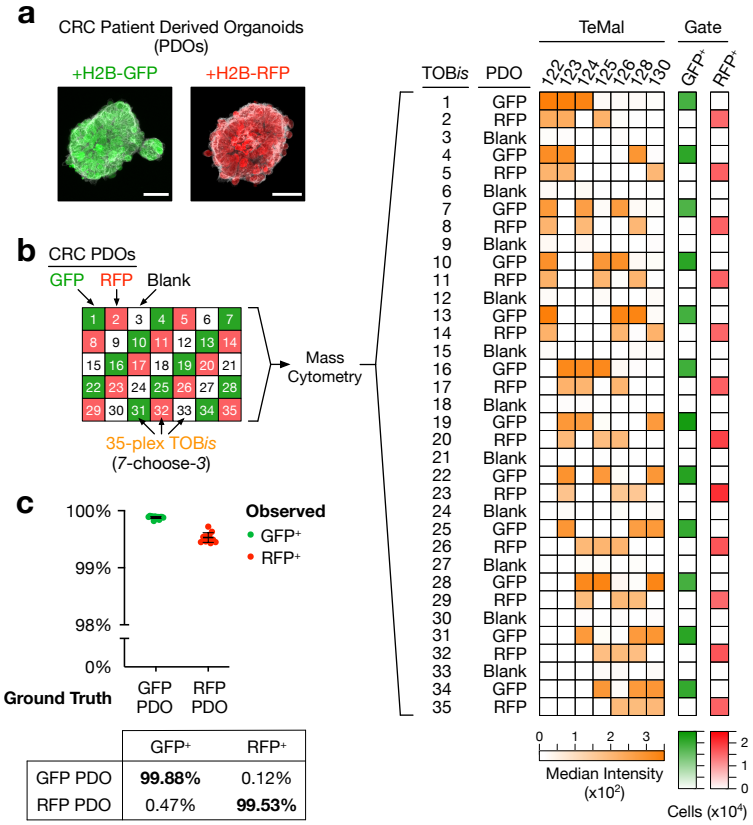

a
Barcodes
TeMal
Thiol Reactive
Metal
Cisplatin
122Te
123Te
124Te
125Te
126Te
128Te
130Te
196Pt
198Pt
35-plex TOBis (7-choose-3)
126-plex TOBis (9-choose-4)
Wash
Glutathione
Thiol
b
Organoid Culture 1
TOBis 1
Organoid Culture 126
TOBis 126
Barcode Organoids in situ
Quench Unbound Barcodes
Pooled Single-Cell Dissociation
Single-Cell Mass Cytometry
Debarcode Organoid Cells
Intensity
m/z
c
35-plex TOBis (7-choose-3)
Median Intensity (x10³)
Barcode 'Off'
Barcode 'On'
Frequency
Barcode Intensity
d
126-plex TOBis (9-choose-4)
Median Intensity (x10³)

Figure 4



	GFP+	RFP+
GFP PDO	**99.88%**	0.12%
RFP PDO	0.47%	**99.53%**

Figure 5

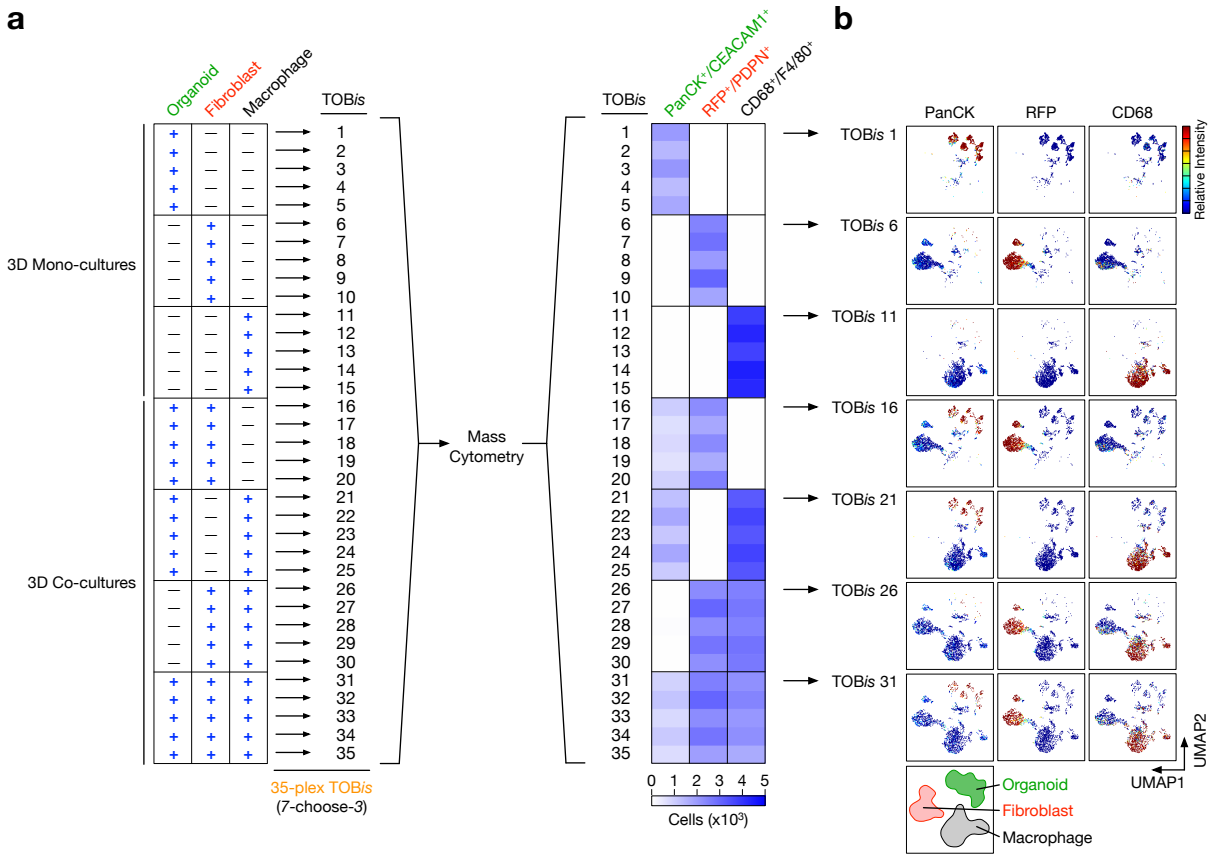

a
Organoid
Fibroblast
Macrophage
TOBis
3D Mono-cultures
3D Co-cultures
35-plex TOBis
(7-choose-3)
Mass
Cytometry
PanCK+/CEACAM1+
RFP+/PDPN+
CD68+/F4/80+
Cells (x10³)
b
PanCK
RFP
CD68
Relative Intensity
TOBis 1
TOBis 6
TOBis 11
TOBis 16
TOBis 21
TOBis 26
TOBis 31
UMAP1
UMAP2
Organoid
Fibroblast
Macrophage

Figure 6

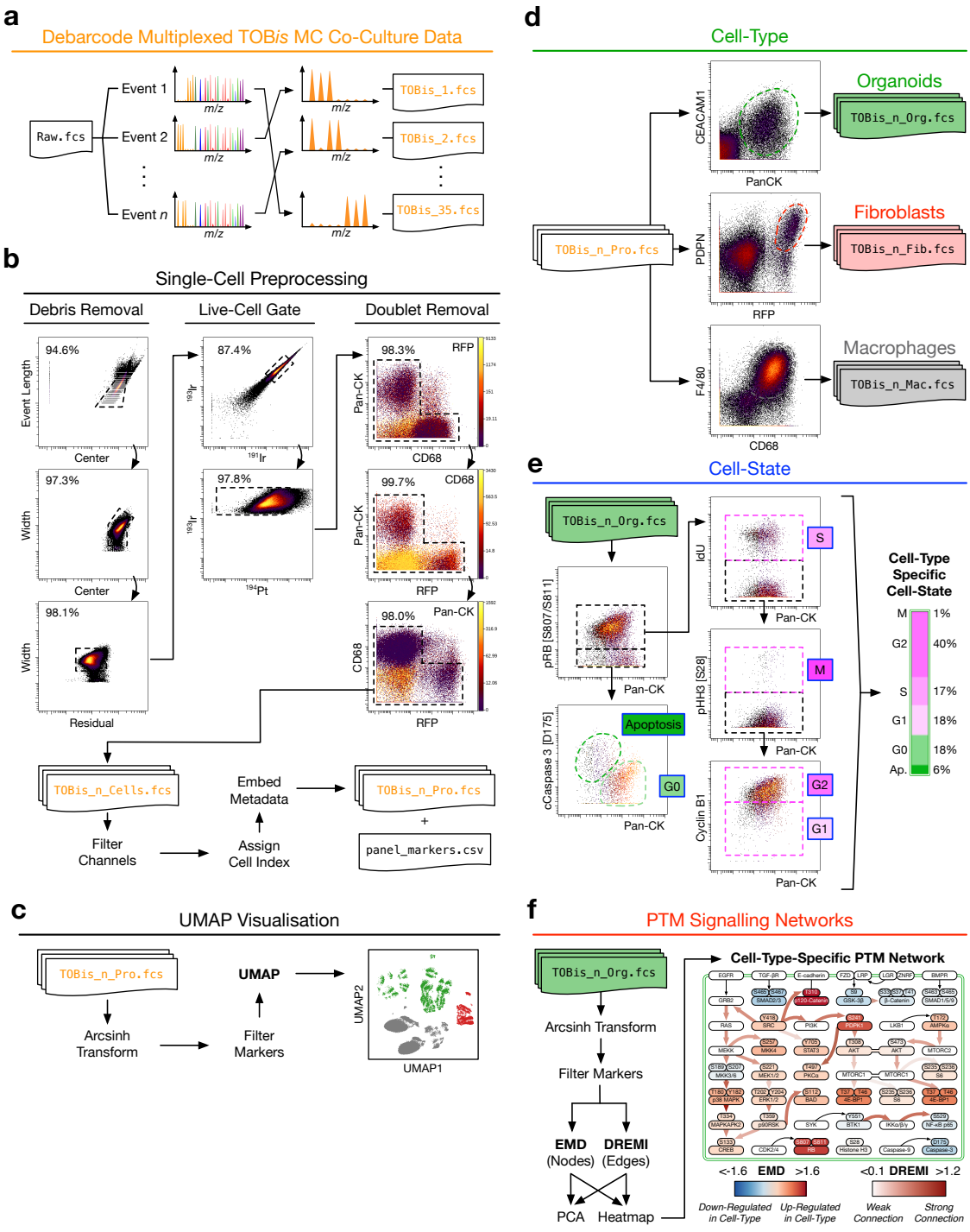

a
Debarcode Multiplexed TOBis MC Co-Culture Data
Raw.fcs
Event 1
Event 2
Event n
m/z
TOBis_1.fcs
TOBis_2.fcs
TOBis_35.fcs
b
Single-Cell Preprocessing
Debris Removal
Live-Cell Gate
Doublet Removal
94.6%
Event Length
Center
97.3%
Width
Center
98.1%
Width
Residual
87.4%
191Ir
97.8%
191Ir
194Pt
98.3%
RFP
Pan-CK
CD68
99.7%
CD68
Pan-CK
RFP
98.0%
Pan-CK
CD68
RFP
TOBis_n_Cells.fcs
Filter Channels
Assign Cell Index
Embed Metadata
TOBis_n_Pro.fcs
+
panel_markers.csv
c
UMAP Visualisation
TOBis_n_Pro.fcs
Arcsinh Transform
UMAP
Filter Markers
UMAP2
UMAP1
d
Cell-Type
TOBis_n_Pro.fcs
CEACAM1
PanCK
Organoids
TOBis_n_Org.fcs
PDPN
RFP
Fibroblasts
TOBis_n_Fib.fcs
F4/80
CD68
Macrophages
TOBis_n_Mac.fcs
e
Cell-State
TOBis_n_Org.fcs
pRB [S807/S811]
Pan-CK
IdU
S
Pan-CK
pHH3 [S28]
M
Pan-CK
cCaspase 3 [D175]
Apoptosis
G0
Pan-CK
Cyclin B1
G2
G1
Pan-CK
Cell-Type Specific Cell-State
M 1%
G2 40%
S 17%
G1 18%
G0 18%
Ap. 6%
f
PTM Signalling Networks
TOBis_n_Org.fcs
Arcsinh Transform
Filter Markers
EMD (Nodes)
DREMI (Edges)
PCA
Heatmap
Cell-Type-Specific PTM Network
<-1.6 EMD >1.6
Down-Regulated in Cell-Type
Up-Regulated in Cell-Type
<0.1 DREMI >1.2
Weak Connection
Strong Connection

FIGURE LEGENDS

Figure 1 – Organoids are High-Dimensional Systems.

Schematic of a small intestinal (SI) organoid. SI organoids contain multiple cell-types, each in a unique cell-state. Each cell contains cell-type- and cell-state-specific post-translational modification (PTM) signalling networks. High-dimensional technologies are needed to simultaneously quantify cell-type and cell-state-specific PTM signalling in organoids.

Figure 2 – TOB*is* MC Protocol Overview.

Live organoids are treated with ^{127}IdU (identifying S-phase cells) and phosphatase and protease inhibitors (inhib.) (Procedure Steps 1 to 3). Organoids are then fixed *in situ* with paraformaldehyde (PFA) and stained with ^{194}Cisplatin (^{194}Cisp) to identify dead / dying cells (Steps 4 to 7). Organoids from different experimental conditions are barcoded with TOB*is* reagents while still in Matrigel (Fig. 3), washed with reduced glutathione (GSH), and pooled (Steps 8 to 10). Organoids are dissociated into single cells using Dispase II, Collagenase IV, and DNase I and stained with extracellular cell-type rare-earth metal conjugated antibodies (Abs) (Steps 11 to 24). Cells are then permeabilised with Triton and Methanol, stained with intracellular post-translational modification (PTM), cell-sate, and cell-type Abs (Steps 25 to 33). Abs are cross-linked to their antigens using formaldehyde (FA) and cells are incubated with $^{191/193}$Iridium (Ir) DNA intercalators (Steps 34 to 37). Single cells are analysed using a mass cytometer (Steps 38 to 49). Different experimental conditions are debarcoded, pre-processed, and single-cell organoid data is visualised using Uniform Manifold Approximation and Projection (UMAP) (Steps 50 to 60). Cell-type-specific PTM node intensity is

calculated using Earth Mover's Distance (EMD) between reference (*R*) and variable (*V*) populations and PTM-PTM connectivity is calculated using Density Resampled Estimation of Mutual Information (DREMI) (Steps 61 to 70). CSB, cell staining buffer (see REAGENTS); TOF-MS, time-of-flight mass spectrometer.

Figure 3 – TOB*is* Multiplexing Overview.

a) TOB*is* reagents. Seven thiol-reactive tellurium maleimide (TeMal) isotopologues (^{122}Te, ^{123}Te, ^{124}Te, ^{125}Te, ^{126}Te, ^{128}Te, and ^{130}Te) are combined to form a doublet-filtering 35-plex (*7*-choose-*3*) barcoding matrix. Two additional Cisplatin isotopologues (^{196}Pt and ^{198}Pt) can expand the barcoding matrix to 126-plex (*9*-choose-*4*). Reduced glutathione provides a source of free thiols to quench unbound barcodes during wash steps.

b) TOB*is* workflow schematic. Ma, maleimide.

c) Histograms of monoisotopic Te (coloured by the median intensity) of 35 murine *shApc* / *Kras*$^{G12D/+}$ CRC organoid cultures barcoded using 35-plex (*7*-choose-*3*) TOB*is* (barcode key provided in Supplementary Table 3). The X axis represents the signal intensities of the corresponding barcodes. For each TOB*is* condition, 3 of the 7 Te isotopes need to be 'on' and the other 4 isotopes need to be 'off' to achieve successful sample demultiplexing.

d) Histograms of monoisotopic Te and Pt (coloured by the median intensity) of 126 murine *shApc* / *Kras*$^{G12D/+}$ CRC organoid cultures barcoded using 126-plex (*9*-choose-*4*) TOB*is* (barcode key provided in Supplementary Table 4). The X axis represents the signal intensities of the corresponding barcodes. For each TOB*is* condition, 4 of the

9 Te / Pt isotopes need to be 'on' and the other 5 isotopes need to be 'off' to achieve successful sample demultiplexing.

Figure 4 – TOB*is* MC Barcoding Fidelity.

a) Confocal microscopy of human colorectal cancer (CRC) patient-derived organoids (PDOs) transfected with either H2B-GFP (endogenous, green) or H2B-RFP (endogenous, red), stained for EpCAM (white); scale bars, 25 μm.

b) Checkerboard plating of GFP PDOs, RFP PDOs, or blank wells with only Matrigel. Cells were barcoded with 35-plex (*7*-choose-*3*) TOB*is* and analysed by MC.

c) Percentage of GFP^+ and RFP^+ positive cells ("Observed") recovered from GFP and RFP PDO cultures ("Ground Truth") (n = 12 independent samples for each PDO). TOB*is* achieved a barcoding accuracy of >99%. Error bars represent standard deviation.

Figure 5 – TOB*is* MC for Organoid Co-cultures.

a) Mono- and co-cultures of organoids, fibroblasts, and macrophages (n = 5) barcoded using 35-plex (*7*-choose-*3*) TOB*is* and analysed by MC. The heatmap shows numbers of organoids (Pan-cytokeratin $(PanCK)^+$ and $CEACAM1^+$), fibroblasts (Podoplanin $(PDPN)^+$ and Red Florescent Protein $(RFP)^+$), and macrophages ($CD68^+$ and $F4/80^+$) recovered from each TOB*is* barcode. TOB*is* MC specifically resolved all the conditions and successfully labelled all the cell-types within the culture.

b) UMAP projections of single cells from representative debarcoded TOB*is* conditions demonstrating the compatibility of TOB*is* to organoid co-cultures.

Figure 6 – TOB*is* MC Data Analysis using CyGNAL.

a) TOB*is* MC raw data is debarcoded[44] into individual experimental conditions (TOBis_n.fcs).

b) All debarcoded data files are imported into a cytometry data analysis platform for debris removal, live-cell identification, and doublet removal (percentages shown are of the parent population), yielding cells for analysis (TOBis_n_Cells.fcs). The data files are then processed by the CyGNAL pipeline[49], which generates pre-processed datasets (TOBis_n_Pro.fcs) and a file containing the list of markers used in the experiment (panel_markers.csv).

c) UMAP[50] dimensionality reduction is performed using user-defined markers.

d) Cell-type identification is performed on pre-processed data to generate cell-type-specific datasets.

e) Cell-state analysis is performed on cell-type-specific data.

f) PTM signalling analysis is performed on cell-type-specific data via EMD[51, 52] and DREMI[53] calculations. EMD and DREMI scores can be visualised in heatmaps or summarised using principal component analysis (PCA).

- Supplementary Information -

Multiplexed Single-Cell Analysis of Organoid Signalling Networks

Jahangir Sufi[1$], Xiao Qin[1$], Ferran Cardoso Rodriguez[1], Yong Jia Bu[2], Petra Vlckova[1], María Ramos Zapatero[1], Mark Nitz[2], Christopher J. Tape[1*]

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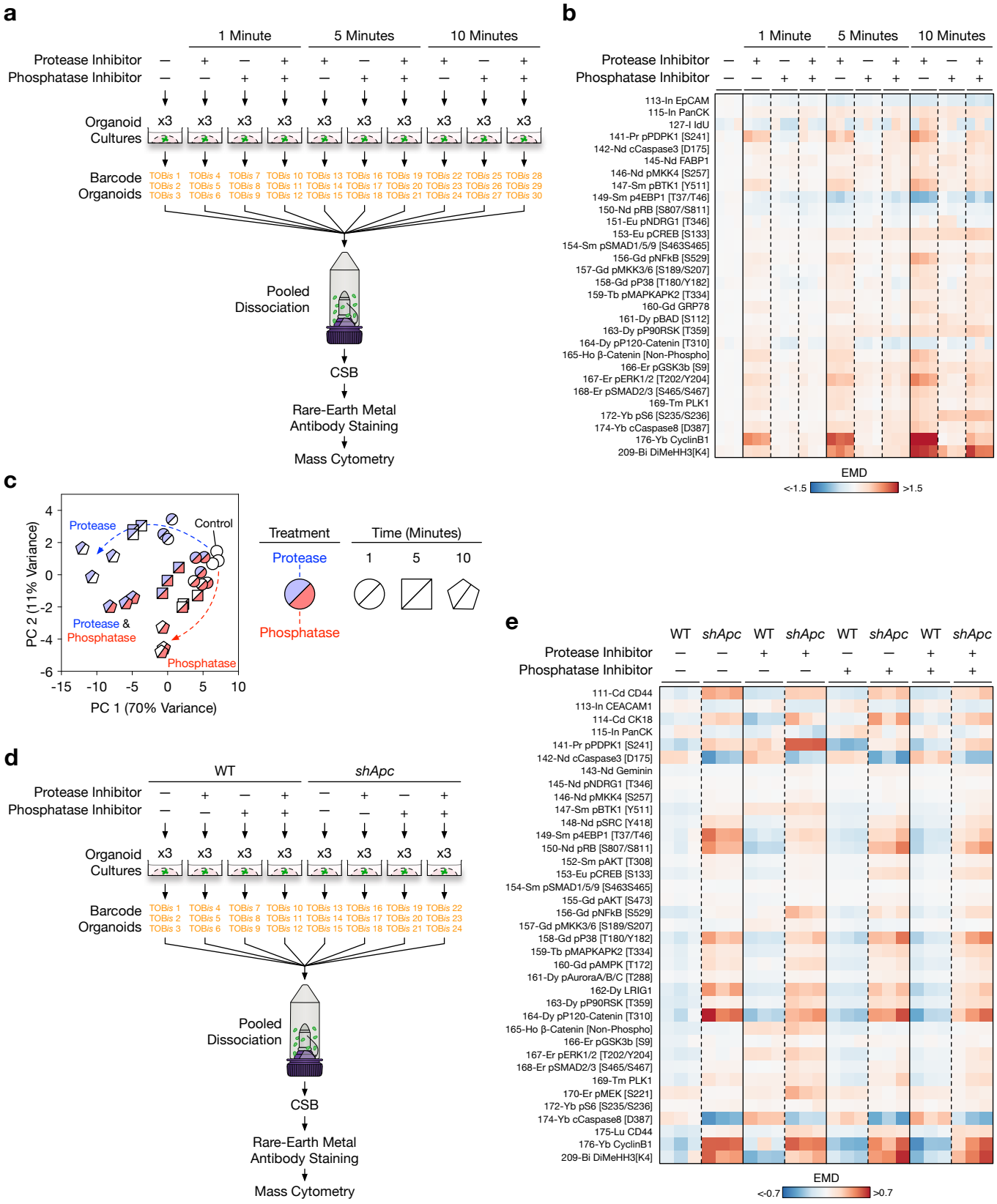
[2] Department of Chemistry, University of Toronto, Toronto, Canada.

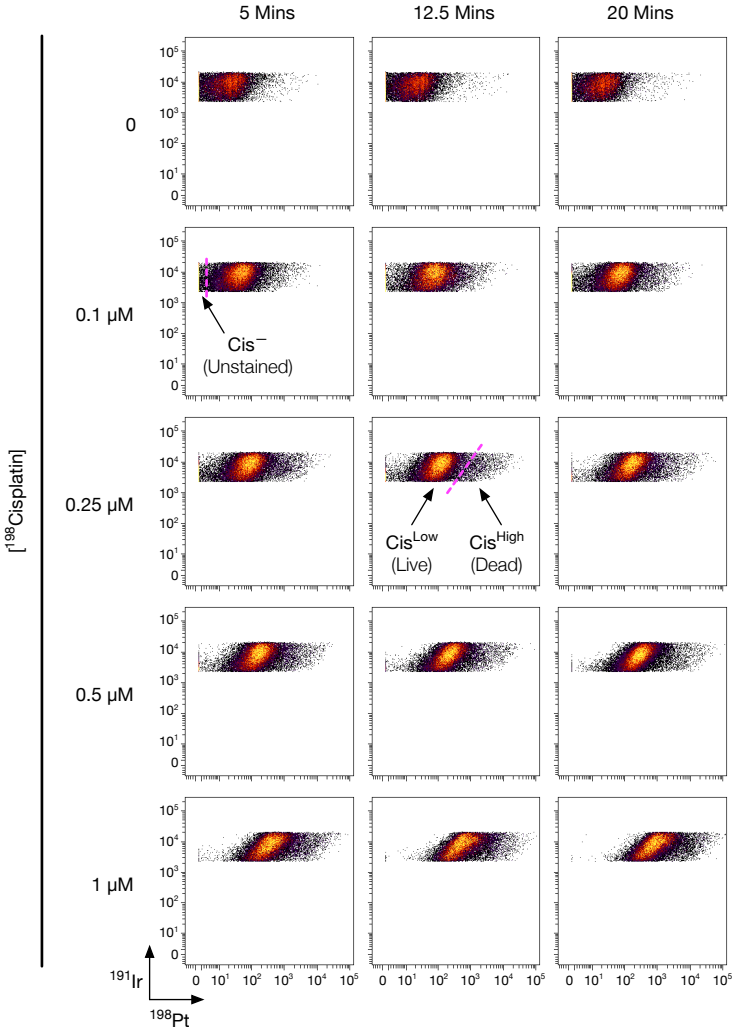
[$]These authors contributed equally to this work.
*Correspondence: c.tape@ucl.ac.uk

Supplementary Figure 1



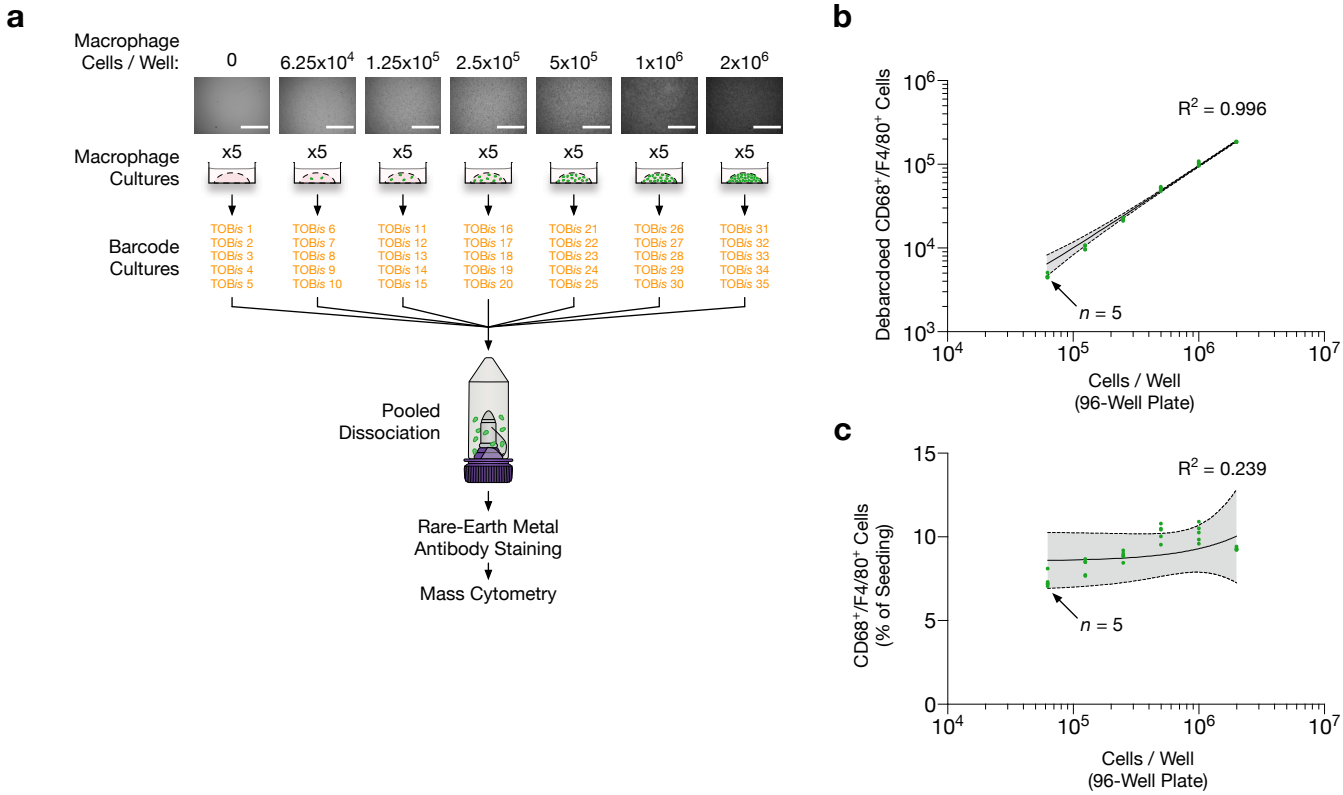
Supplementary Figure 1 – Protease and Phosphatase Inhibitor Pre-Treatment. a) Experimental overview. WT murine small intestinal organoids were treated with either protease inhibitors, phosphatase inhibitors, or both for 1, 5, or 10 minutes before fixation (n = 3). All conditions were TOB*is* barcoded, pooled for dissociation, stained with rare earth metal antibodies, and analysed by mass cytometry (MC). b) Earth Mover's Distance (EMD) heatmap of MC parameters following different organoid pre-treatments. Both protease and phosphatase inhibitor pre-treatments can alter heavy-metal antibody staining. c) Principal component analysis (PCA) of data in b). Protease and phosphatase inhibitor pre-treatment affects heavy-metal antibody staining in a time-dependent manner. Users are advised to empirically determine the duration of the treatment according to their experimental system and antibody panel. d) Experimental overview. Murine colonic organoids with either wild-type (WT) or Apc knockdown (*shApc*) were pre-treated for 5 minutes with either protease inhibitors, phosphatase inhibitors, or both, TOB*is* barcoded, dissociated, stained with rare-earth metal antibodies, and analysed by mass cytometry (MC). e) EMD heatmap of MC parameters following different inhibitor pre-treatments. Note how different inhibitor pre-treatments can alter antigen staining (e.g., pPDK1 [S241], pNFkB [S529]). CSB, cell staining buffer (see REAGENTS).

Supplementary Figure 2



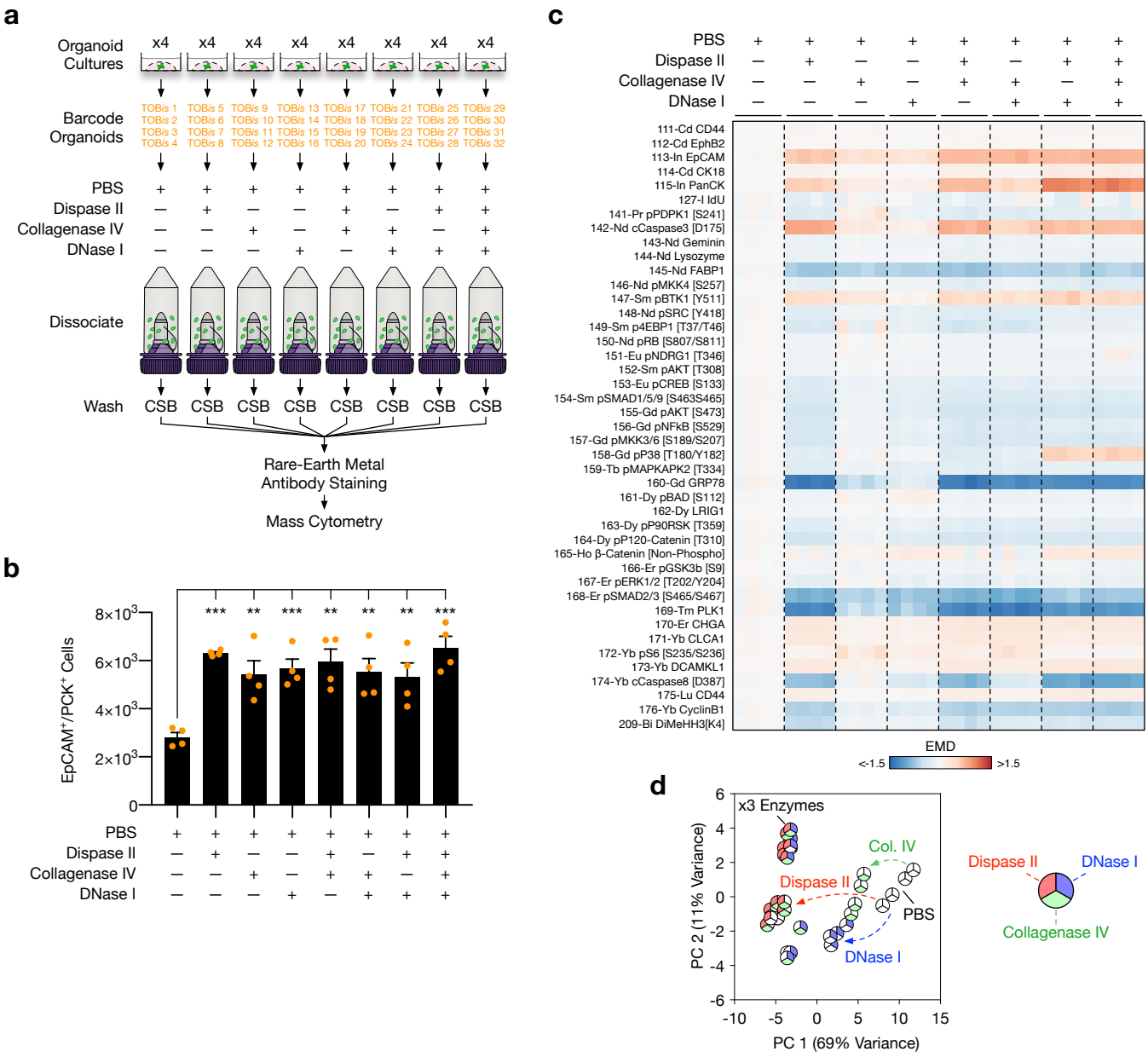
Supplementary Figure 2 – Cisplatin Organoid Staining *in situ*. 0 – 1 μM ^{198}Cisplatin was added to murine small intestinal organoids over 5, 12.5, and 20 minutes and analysed by mass cytometry. Cisplatin^{Low} cells can be retained as live cells and Cisplatin^{High} cells can be gated out as dead cells. We advise users to select a combination of ^{198}Cisplatin concentration and incubation time where all cells have had the opportunity to be stained with Cisplatin (i.e., no Cisplatin^{-} cells). CSB, cell staining buffer (see REAGENTS).

Supplementary Figure 3



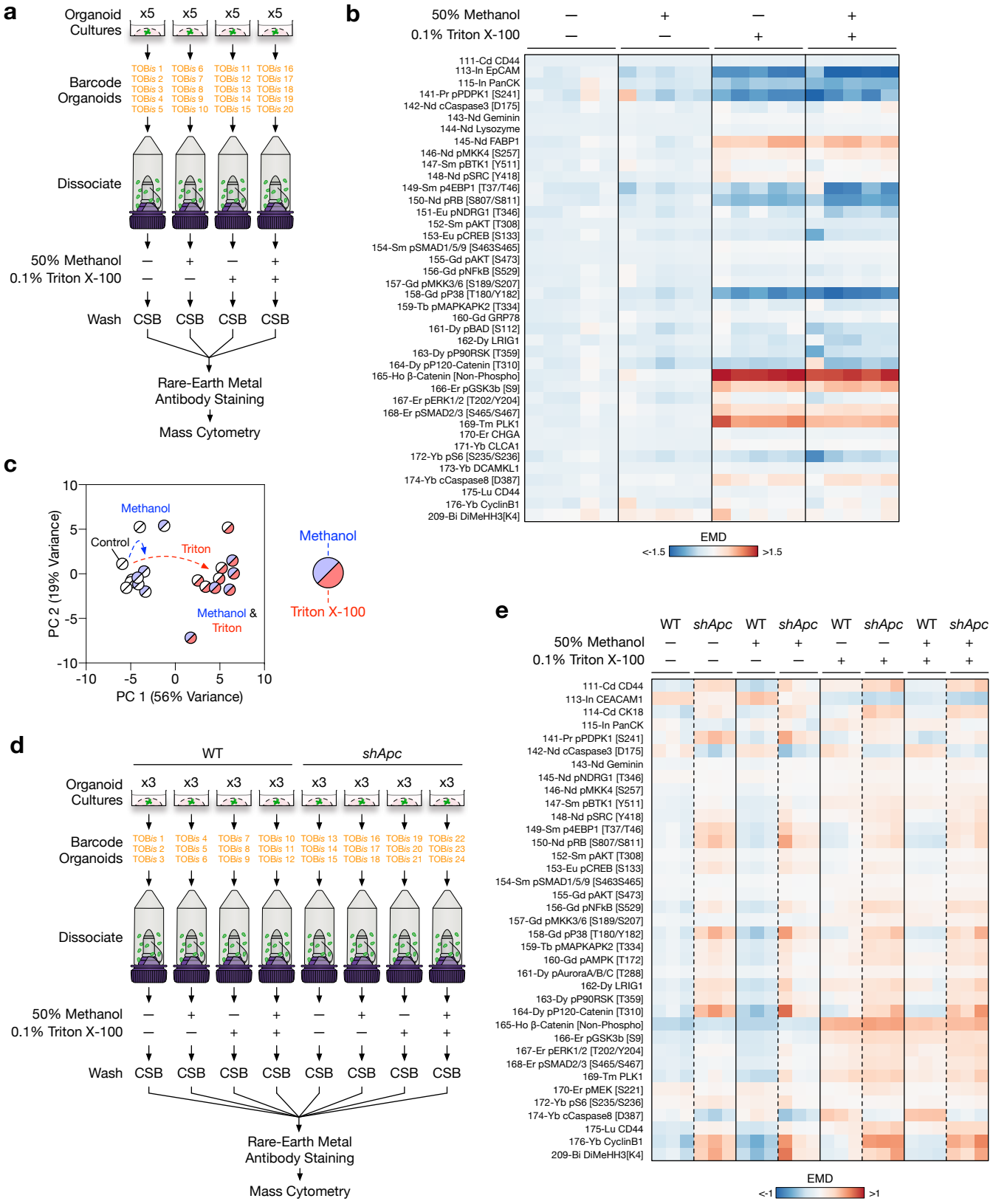
Supplementary Figure 3 – TOB*is* Barcoding Capacity. a) Experimental overview. Bone marrow derived macrophages (BMDMs) were plated in 96-well plates ranging from $6.25x10^4$ – $2x10^6$ cells per well in 3D Matrigel (n = 5). (Note: a typical seeding density for cells in 3D Matrigel in a 96-well plate is $<1x10^6$ per well). Cells were TOB*is* barcoded, dissociated, stained with rare-earth metal antibodies, and analysed by mass cytometry. Scale bar = 1 mm. b) and c) Debarcoded $CD68^+$ /$F4/80^+$ BMDMs display linear debarcoding recovery across all cell densities. Error represents 95% confidence interval (area shaded grey).

Supplementary Figure 4



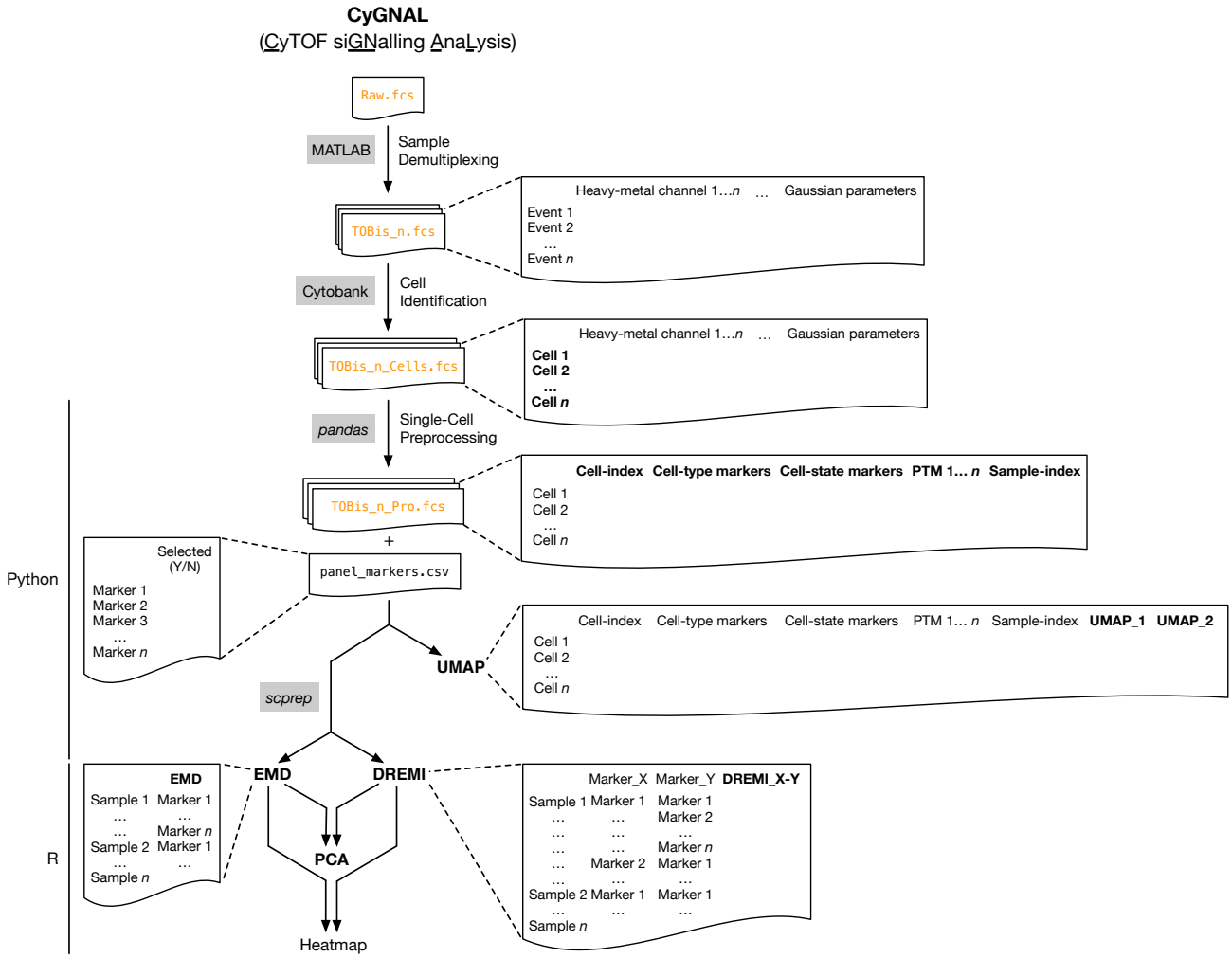
Supplementary Figure 4 – Single-Cell Organoid Dissociation Enzymes. a) Experimental overview. Murine small intestinal organoids were TOB*is* barcoded and dissociated in PBS supplemented with either Dispase II, Collagenase IV, and/or DNase I (n = 4) using the Miltenyi GentleMACS platform, stained with rare-earth metal antibodies, and analysed by mass cytometry (MC). b) Debarcoded $EpCAM^+$ /PCK^+ cells from each dissociation condition. All enzymes improve single-cell recovery relative to PBS alone (2-tailed unpaired *t*-test vs PBS, ** = $p<0.01$, *** = $p<0.001$). c) Earth Mover's Distance (EMD) heatmap of MC parameters following different dissociation conditions. Each dissociation enzyme can alter heavy-metal antibody staining and should be optimised for each biological system studied. d) Principal component analysis (PCA) of data in c). Heavy-metal antibody staining is affected by the combination of dissociation enzymes, and users are advised to test and titrate alternative dissociation enzymes for their experimental system. CSB, cell staining buffer (see REAGENTS).

Supplementary Figure 5



Supplementary Figure 5 – Cell Permeabilisation Buffers. a) Experimental overview. Murine small intestinal organoids were TOB*is* barcoded (n = 5), dissociated, and permeabilised using 50% methanol, 0.1% Triton, or both, stained with rare-earth metal antibodies, and analysed by mass cytometry (MC). b) Earth Mover's Distance (EMD) heatmap of MC parameters following different permeabilisation conditions. In our experience, 0.1% Triton is a more effective permeabilisation buffer for small intestinal organoids, but this may vary with alternative models and should be optimised by for each biological system studied. c) Principal component analysis (PCA) of data in b). Permeabilisation conditions can substantially alter heavy-metal antibody staining, and users are advised to optimise the step based on their model system and antibody panel. d) Experimental overview. Wild-type (WT) or Apc knockdown (*shApc*) murine colonic organoids were TOB*is* barcoded, dissociated, and permeabilised using 50% methanol, 0.1% Triton, or both (n = 3), stained with rare-earth metal antibodies, and analysed by mass cytometry (MC). e) EMD heatmap of MC parameters following different permeabilisation conditions. In murine colonic organoids 50% methanol treatment yields similar antibody staining to untreated cells. Either 0.1% Triton alone or combined with 50% methanol yields strong staining across multiple parameters. CSB, cell staining buffer (see REAGENTS).

Supplementary Figure 6



Supplementary Figure 6 – CyGNAL Architecture. File structure and processing architecture of CyTOF siGNalling AnaLysis (CyGNAL) pipeline for computing cell-type-specific signalling networks from TOB*is* MC data. Software environments and packages are indicated in grey boxes, computational processes are in bold text.

Supplementary Table 1 – TOB*is* MC Costs.

	Item	Price / Well (£)	35-plex TOB*is*	126-plex TOB*is*
Pre-Treatment	^{127}IdU	0.15	5.25	18.9
	Protease Inhibitors	0.035	1.23	4.41
	Phosphatase Inhibitors	0.078	2.73	9.83
	PFA	0.0084	0.29	1.1
	^{194}Cisplatin	0.088	3.08	11.09
TOB*is*	TeMal *	0.06 or 0.08	2.1	10.08
	$^{196/8}$Cisplatin **	0.005	-	0.63
		Price / C-Tube (£)	**x5 C-Tubes**	**x15 C-Tubes**
Dissociation	C-Tubes	3.9	19.5	58.5
	Dispase II	0.15	0.75	2.25
	Collagenase IV	0.16	0.8	2.4
	DNase I	0.18	0.9	2.7
			Price / 3M Cell Stain (£)	**Price / 12M Cell Stain (£)**
Staining	40-plex Metal-Antibody Panel ***		120	480
	^{191}Ir + ^{193}Ir		0.18	0.72
		Total (£)	156.81	602.61

Costing guidelines for multiplexed TOB*is* MC experiments. Metal-antibody conjugates comprise 75-80% of the total cost of a TOB*is* MC experiment.

* Based on TeMal production in an academic lab (0.3 mmole = £2,000).

** Based on custom production of 196/8 Cisplatin from BuyIsotope (1 mL 10 mM = £1000).

*** Based on 100 ug Metal-antibody = £300 (£200 Ab, £100 conjugation), 1 ug / Metal-Ab / 3 million cell staining.

Supplementary Table 2 – Murine Small Intestinal Organoid Mass Cytometry Antibody Panel.

Isotope-Metal	Antigen / Target	Antibody Clone	Supplier	Catalogue #
89-Y	Phospho-Histone H3 [S28]	HTA28	BioLegend	641007
113-In	CD326 (EpCAM)	G8.8	BioLegend	118223
115-In	Pan-Cytokeratin (Pan-CK)	AE-1/AE-3	BioLegend	914204
141-Pr	Phospho-PDPK1 [S241]	J66-653.44.22	BD Biosciences	558395
142-Nd	Cleaved-Caspase 3 [D175]	D3E9	CST	9579
143-Nd	C-MYC	D84C12	CST	5605
144-Nd	Lysozyme	BGN/06/961	Abcam	ab36362
145-Nd	FABP1	328605	R&D Systems	MAB29641
146-Nd	Phospho-MKK4/SEK1 [S257]	C36C11	CST	4514
147-Sm	Phospho-BTK [Y551]	24a/BTK	BD Biosciences	558034
148-Nd	Phospho-SRC [Y418]	SC1T2M3	Thermo	14-9034-82
149-Sm	Phospho-4E-BP1 [T37/46]	236B4	CST	2855
150-Nd	Phospho-RB [S807/811]	J112-906	BD Biosciences	558389
151-Eu	Phospho-PKCα [T497]	K14-984	BD Biosciences	610108
152-Sm	Phospho-AKT [T308]	J1-223.371	BD Biosciences	558316
153-Eu	Phospho-CREB [S133]	87G3	CST	9198
154-Sm	Phospho-SMAD1 [S463/465] Phospho-SMAD5 [S463/465] Phospho-SMAD9 [S465/467]	D5B10	CST	13820
155-Gd	Phospho-AKT [S473]	D9E	CST	4060
156-Gd	Phospho-NF-κB p65 [S529]	K10-895.12.50	BD Biosciences	558393
157-Gd	Phospho-MKK3 [S189] / MKK6 [S207]	D8E9	CST	12280
158-Gd	Phospho-p38 MAPK [T180/Y182]	D3F9	CST	4511
159-Tb	Phospho-MAPKAPK2 [T334]	27B7	CST	3007
160-Gd	Phospho-AMPKα [T172]	40H9	CST	2535
161-Dy	Phospho-BAD [S112]	40A9	CST	5284
162-Dy	LRIG1	Polyclonal	R&D Systems	AF3688
163-Dy	Phospho-p90RSK [T359]	D1E9	CST	8753
164-Dy	Phospho-p120-Catenin [T310]	22/p120 (pT310)	BD Biosciences	558203
165-Ho	β-Catenin [Active]	D13A1	CST	8814
166-Er	Phospho-GSK-3β [S9]	D85E12	CST	5558
167-Er	Phospho-ERK1/2 [T202/Y204]	20A	BD Biosciences	612359
168-Er	Phospho-SMAD2 [S465/467] Phospho-SMAD3 [S423/425]	D27F4	CST	8828
169-Tm	GFP	5F12.4	Fluidigm	3169009
170-Er	Phospho-MEK1/2 [S221]	166F8	CST	2338
171-Yb	CLCA1	EPR12254-88	Abcam	ab180851
172-Yb	Phospho-S6 [S235/236]	D57.2.2E	CST	4858
173-Yb	DCAMKL1	6F9	Sigma	WH0009201M2
174-Yb	CHR-A	C-12	Santa Cruz	sc-393941
175-Lu	CD44	IM7	BioLegend	103051
176-Yb	Cyclin B1	GNS-11	BD Biosciences	554179
209-Bi	Di-Methyl-Histone H3 [K4]	C64G9	CST	9725

Extracellular
Intracellular

SUPPLEMENTARY METHOD

Synthesis and Characterization of TeMal Isotopologues

REAGENTS

(Triisopropylsilyl)acetylene (Sigma, Cat# 360031)

Acetone (Sigma, Cat# 179124)

N-bromosuccinimide (Sigma, Cat# B81255)

Silver(I) nitrate (Sigma, Cat# 209139)

Pentane (Sigma, Cat# 158941)

Sodium chloride (Sigma, Cat# S9888)

Sodium sulfate (Sigma, Cat# 238597)

n-Butylamine (Sigma, Cat# 471305)

Copper(I) chloride (Sigma, Cat# 224332)

Hydroxylamine hydrochloride (Sigma, Cat# 159417)

4-pentynoic acid (Sigma, Cat# 232211)

Ethyl acetate (Sigma, Cat# 319902)

Citric acid (Sigma, Cat# C0759)

Magnesium sulfate (Sigma, Cat# 746452)

Isotopically enriched tellurium (Trace Sciences International, custom order)

- ^{122}Te – tellurium metal powder
- ^{123}Te – tellurium oxide
- ^{124}Te – tellurium metal powder
- ^{125}Te – tellurium metal powder
- ^{126}Te – tellurium metal powder

^{128}Te – tellurium metal powder

^{130}Te – tellurium metal powder

Hydrazine hydrate (Sigma, Cat# 225819)

Sodium borohydride (Sigma, Cat# 452882)

Tetrahydrofuran (THF) (Sigma, Cat# 401757)

Tetrabutylammonium fluoride, 1.0 M in THF (TBAF) (Sigma, Cat# 216143)

Ammonium chloride (Sigma, Cat# A9434)

Diethyl ether (Sigma, Cat# 673811)

Ethanol (Greenfield Global, Cat# P210EAAN)

Methanol (Sigma, Cat# 179337)

Glacial acetic acid (Caledon Laboratory Chemicals, Cat# 1000-1-29)

Dichloromethane (Sigma, Cat# D65100)

1-Hydroxybenzotriazole monohydrate (HOBt, TCI America, Cat# H0468)

N,N-diisopropylethylamine (DIPEA, Sigma, Cat# D125806)

HATU (Sigma, Cat# 445460)

N-(2-Aminoethyl)maleimide hydrochloride (TCI America, Cat# A2436)

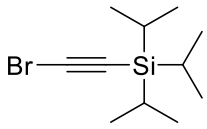
Sodium bicarbonate (Sigma, Cat# S6014)

Ammonium hydroxide (Caledon Laboratory Chemicals, Cat# 1525-1-29)

SiliaFlash silica gel P60 (Silicycle, Cat# R12030B)

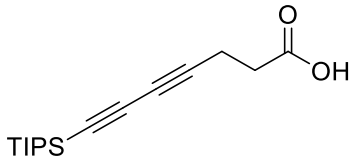
Synthesis and Characterization of TeMal Isotopologues

Reagents and solvents described below were purchased from Sigma-Aldrich, TCI America, Caledon laboratory Chemicals, or Greenfield Global (see reagents) and used as supplied unless otherwise indicated. Isotopically enriched tellurium metal was purchased from Trace Sciences. Solvents were degassed by sparging argon through vessels under sonication for at least one hour. Column chromatography was performed using SiliaFlash P60 (Silicycle); a combination of gravity elution and moderate air pressure was employed. NMR spectra were acquired using a 500 MHz Agilent DD2 spectrometer with an XSens C13 Cold Probe or a 400 MHz Bruker Avance III spectrometer with a dual resonance (BBFO) broad band probe. Mass spectra were obtained by positive mode electrospray (ESI+) on an Agilent 6538 Q-TOF mass spectrometer.

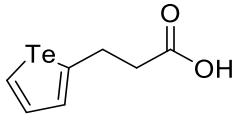


(Bromoethynyl)triisopropylsilane (1). This is a commercially available reagent but can easily be synthesized in-house. To a 250 mL round bottom flask was added acetone (65 mL), TIPS-acetylene (1.9 mL, 8.2 mmol, 1.0 eq), *N*-bromosuccinimide (1.47 g, 8.26 mmol, 1.0 eq), and catalytic silver nitrate (0.136 g, 0.80 mmol, 0.1 eq). The mixture was stirred at room temperature (observed formation of small amounts of white precipitate). After 3 hr, pentanes (~130 mL) was added and the mixture stirred for 30 min to precipitate succinimide and salts. The mixture was gravity filtered into a separatory funnel and the organic filtrate was washed with deionized water (4 x 50 mL) and brine (1 x 50 mL). The remaining organic layer was dried over sodium sulfate, gravity filtered, and concentrated by rotary evaporation until a clear oil remained. The oil was further dried on a high vacuum pump to remove pentanes. As the product is volatile, care was taken not to keep the product under high vacuum for prolonged periods of time or significant losses would occur. Final product was a clear, very pale-yellow oil (1.62 g, 76%). ^{1}H NMR ($CDCl_3$, 500 MHz): δH 1.12-1.03 (21H, mult, Si-***CH***-

$(CH_3)_2$ and Si-CH-(***CH₃***)$_2$). $^{13}C\{^1H\}$ NMR ($CDCl_3$, 125 MHz): δC 83.61 (s, Br-***C***≡C-Si-); 61.87 (s, Br-C≡***C***-Si-); 18.63 (s, Si-CH-(***CH₃***)$_2$); 11.40 (s, Si-***CH***-$(CH_3)_2$).



7-(Triisopropylsilyl)hepta-4,6-diynoic acid (2). To a 50 mL RBF on ice was added degassed, deionized H_2O (17.5 mL), *n*-butylamine (8.5 mL), and CuCl (0.039 g, 0.39 mmol, 0.05 eq) which generated a deep blue colour. $NH_2OH{\cdot}HCl$ was added until reaction changed from blue to colourless (~0.06 g). 4-pentynoic acid (0.870 g, 8.87 mmol, 1.1 eq) was added and the reaction stirred until solids were completely dissolved. The flask was purged with argon and (bromoethynyl)triisopropylsilane (2.04 g, 7.85 mmol, 1.0 eq) was delivered in neat form by syringe; an emulsion was formed which cleared to give a pale-yellow solution after vigorous stirring. After 1.5 hr, the reaction mixture was diluted with deionized water to ~50 mL and extracted with 2 x 50 mL EtOAc. The aqueous phase was acidified with 5% citric acid (~40 mL) and extracted with 2 x 50 mL EtOAc. The combined organic phase was washed with 3 x 40 mL 5% citric acid and 2 x 40 mL brine, dried over $MgSO_4$, and concentrated by rotary evaporation. This yielded a clear yellow oil which formed white crystals upon drying under high vacuum (1.90 g, 6.7 mmol, 91%). ^1H NMR ($CDCl_3$, 400 MHz): δH 2.62 (4H, s, COOH-***CH₂***-CH_2- and COOH-CH_2-***CH₂***-), 1.07 (21H, s, Si-***CH***-$(CH_3)_2$ and Si-CH-(***CH₃***)$_2$). $^{13}C\{^1H\}$ NMR ($CDCl_3$, 100 MHz): δC 177.26 (s, COOH); 89.74, 81.41, 75.98, 66.85 (s, four *sp* carbons); 32.68 (s, COOH-***CH₂***-CH_2-), 18.68 (s, Si-***CH***-$(CH_3)_2$), 15.02 (s, (COOH-CH_2-***CH₂***-), 11.40 (s, Si-CH-(***CH₃***)$_2$).



3-(Tellurophen-2-yl)propanoic acid (3). Where isotopically enriched tellurium samples were obtained in an oxidized state, the precursor was stirred overnight at room temperature in hydrazine hydrate (50-60% hydrazine in H_2O, 5mL per 100 mg tellurium). Contents were dried by careful rotary evaporation to yield a coating of

tellurium (0) which was then directly used in the cyclization reaction. The cyclization reaction and the TIPS-deprotection reaction were run concurrently. The deprotected diyne is somewhat unstable and thus no attempts were made to purify or characterize it before use in the cyclization reaction.

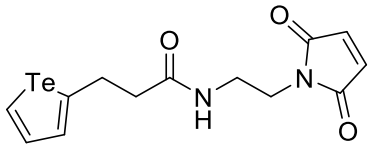
Cyclization Reaction: To a 10 mL RBF was added freshly powdered, isotopically enriched tellurium metal (0.100 g, 0.78 mmol, 1.0 eq) and sodium borohydride (0.237 g, 6.27 mmol, 8.0 eq). The flask was purged using argon for 15 min. Degassed water (3.0 mL) was injected into the flask and the reaction was stirred at room temperature for 1 hr, then heated to 40°C. The reduction of tellurium metal results in a series of colour changes from grey to light purple to very deep purple, then back to light purple, to pink/colourless. As soon as the cyclization reaction was set up, the deprotection reaction was begun.

Deprotection Reaction: To a dry 10 mL RBF under argon, on ice, was added **2** (0.284 g, 1.02 mmol, 1.3 eq. to Te metal) in THF (1.7 mL). TBAF (1.7 mL 1M solution in THF) was injected slowly. The mixture was allowed to stir on ice for 1 hour, after which the reaction mixture was partitioned over 5 mL saturated NH_4Cl and 5 mL diethyl ether. The aqueous layer was extracted with 5mL ether, acidified with ~2.5 mL 5% citric acid (to reach pH ~4) and extract two more times with 5 mL diethyl ether. The combined organic phase as washed with 3 mL 5% citric acid, 3 mL brine, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation to yield a pale yellow/orange oil. The flask containing TIPS-deprotected **2** was sealed and purged with argon. Contents were dissolved in degassed ethanol (3.0 mL) and transferred by syringe into the cyclization reaction.

Injection of diyne into the tellurium mixture resulted in darkening of reaction colour to purplish brown. After stirring for ~10-20 min, the reaction turned a peachy yellow colour. The mixture was stirred overnight at 40°C. After 15 hr, the cyclization reaction was opened to atmosphere and stirred for 1 hour to precipitate unreacted tellurium. Contents were filtered over a small celite column and the filter cake rinsed with minimal amounts of methanol. 5% citric acid was added to the filtrate until bubbling stopped and solution was acidic (pH~3-4). The mixture was extracted with ethyl acetate (3 x 10 mL). The combined organic phase was washed with 10 mL 5% citric

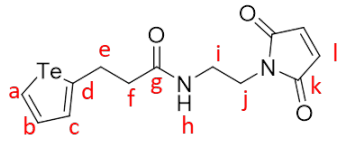
acid, 1mL brine, dried over $MgSO_4$, filtered, and concentrated by rotary evaporation. Crude was purified by column chromatography using isocratic elution in 20% ethyl acetate in pentanes + 0.1% acetic acid. Due to "tail" of product co-eluting with an impurity, some samples were not completely purified (yellow oil containing traces of TIPS-based impurity; some colourless crystals formed upon cooling; NMR yield 45 to 70%).

Non-NMR active Te isotopologues ($^{122, 124, 126, 128, 130}$Te): ^{1}H NMR ($CDCl_3$, 400 MHz): δH 8.72 (1H, dd, $^{3}J_{HH}$ = 6.9 Hz, $^{4}J_{HH}$ = 1.3 Hz, -Te-***CH***-CH-), 7.59 (1H, dd, $^{3}J_{HH}$ = 6.9, 3.9 Hz, -Te-CH-***CH***-), 7.45-7.33 (1H, m, -Te-CR-***CH***-), 3.24 (2H, t, $^{3}J_{HH}$ = 7.3 Hz, COOH-CH_2-***CH_2***-), 2.73 (2H, t, $^{3}J_{HH}$ = 7.3 Hz, COOH-***CH_2***-CH_2-). NMR active Te isotopologues: ^{123}Te: ^{1}H NMR ($CDCl_3$, 400 MHz): δH 8.72 (1H, ddd, $^{2}J_{HTe}$ = 82.8 Hz, $^{3}J_{HH}$ = 6.9 Hz, $^{4}J_{HH}$ = 1.2 Hz, -Te-***CH***-CH-), 7.59 (1H, ddd, $^{3}J_{HTe}$ = 15.1 Hz, $^{3}J_{HH}$ = 6.9, 3.9 Hz, -Te-CH-***CH***-), 7.45-7.33 (1H, m, -Te-CR-***CH***-), 3.30-3.17 (2H, m, COOH-CH_2-***CH_2***-), 2.73 (2H, t, $^{3}J_{HH}$ = 7.3 Hz, COOH-***CH_2***-CH_2-). ^{125}Te: ^{1}H NMR ($CDCl_3$, 400 MHz): δH 8.72 (1H, ddd, $^{2}J_{HTe}$ = 99.8 Hz, $^{3}J_{HH}$ = 6.9 Hz, $^{4}J_{HH}$ = 1.2 Hz, -Te-***CH***-CH-), 7.59 (1H, ddd, $^{3}J_{HTe}$ = 18.1 Hz, $^{3}J_{HH}$ = 6.9, 3.8 Hz, -Te-CH-***CH***-), 7.45-7.33 (1H, m, -Te-CR-***CH***-), 3.39-3.05 (2H, m, COOH-CH_2-***CH_2***-), 2.73 (2H, t, $^{3}J_{HH}$ = 7.3 Hz, COOH-***CH_2***-CH_2-).



TeMal (4). To a 25 mL RBF was added DCM (2.1 mL), **2** (0.050 g, 0.20 mmol, 1.0 eq), HOBt (0.097 g, 0.72 mmol, 3.5 eq), DIPEA (0.22 mL, 1.3 mmol, 6.5 eq), and HATU (0.082 g, 0.22 mmol, 1.1 eq); contents were stirred for 10 minutes until completely dissolved. *N*-(2-aminoethyl)maleimide hydrochloride salt (0.037 g, 0.21 mmol, 1.0 eq) was added to begin the reaction. After stirring 3 hr at room temperature, the reaction mixture was diluted with DCM and concentrated to near dryness by rotary evaporation. The crude was redissolved in 7.5 mL EtOAc, washed with 1.5 mL 5% citric acid, 1 mL deionized H_2O, 1.5 mL saturated $NaHCO_3$, 1 mL deionized H_2O, and 1.5 mL brine. The organic phase was dried over MgSO4, concentrated by rotary

evaporation, and further purified by column chromatography using NH_4OH-pretreated silica (gradient elution of 50→60% EtOAc in pentanes). Isolated yields for isotopologues ranged between 0.040 to 0.063g; 53 to 81%.



Isotopologues with non-NMR active Te (**122, 124, 126, 128, 130-TeMal**): ^{1}H NMR ($CDCl_3$, 500 MHz): δH 8.68 (1H, dd, $^{3}J_{HH}$ = 6.9 Hz, $^{4}J_{HH}$ = 1.2 Hz, H^{a}), 7.56 (1H, dd, $^{3}J_{HH}$ = 6.9, 3.9 Hz, H^{b}), 7.40-7.31 (1H, m, H^{c}), 6.70 (2H, s, H^{l}), 5.83 (1H, br s, H^{h}), 3.78-3.59 (2H, m, H^{j}), 3.46 (2H, q, $^{3}J_{HH}$ = 5.6 Hz, H^{i}), 3.20 (2H, td, $^{3}J_{HH}$ = 7.1 Hz, $^{4}J_{HH}$ = 1.1 Hz, H^{e}), 2.47 (2H, t, $^{3}J_{HH}$ = 7.1 Hz, H^{f}). $^{13}C\{^{1}H\}$ NMR ($CDCl_3$, 125 MHz): δC 172.16 (s, C^{g}), 171.03 (s, C^{k}), 149.24 (s, C^{d}), 136.98 (s, C^{b}), 135.66 (s, C^{c}), 134.35 (s, C^{l}), 125.04 (s, C^{a}), 39.91 (s, C^{f}), 39.18 (s, C^{i}), 37.61 (s, C^{j}), 32.37 (s, C^{e}).

123 TeMal: ^{1}H NMR ($CDCl_3$, 500 MHz): δH 8.67 (1H, ddd, $^{2}J_{HTe}$ = 81.1 Hz, $^{3}J_{HH}$ = 6.9 Hz, $^{4}J_{HH}$ = 1.2 Hz, H^{a}), 7.57 (1H, ddd, $^{3}J_{HTe}$ = 14.3 Hz, $^{3}J_{HH}$ = 6.9, 3.9 Hz, H^{b}), 7.42-7.29 (1H, m, H^{c}), 6.70 (2H, s, H^{l}), 5.80 (1H, br s, H^{h}), 3.78-3.58 (2H, m, H^{j}), 3.46 (2H, q, $^{3}J_{HH}$ = 5.6 Hz, H^{i}), 3.20 (2H, dt, $^{3}J_{HTe}$ = 14.2 Hz, $^{3}J_{HH}$ = 7.1 Hz, H^{e}), 2.47 (2H, t, $^{3}J_{HH}$ = 7.1 Hz, H^{f}). $^{13}C\{^{1}H\}$ NMR ($CDCl_3$, 125 MHz): δC 172.16 (s, C^{g}), 171.03 (s, C^{k}), 149.24 (d, $^{1}J_{CTe}$ = 251.2 Hz, C^{d}), 136.98 (d, $^{2}J_{CTe}$ = 4.3 Hz, C^{b}), 135.66 (s, C^{c}), 134.35 (s, C^{l}), 125.04 (d, $^{1}J_{CTe}$ = 247.6 Hz, C^{a}), 39.91 (d, $^{3}J_{CTe}$ = 5.0 Hz, C^{f}), 39.18 (s, C^{i}), 37.61 (s, C^{j}), 32.37 (d, $^{2}J_{CTe}$ = 25.8 Hz, C^{e}).

125 TeMal: ^{1}H NMR ($CDCl_3$, 500 MHz): δH 8.67 (1H, ddd, $^{2}J_{HTe}$ = 97.8 Hz, $^{2}J_{HH}$ = 6.9 Hz, $^{4}J_{HH}$ = 1.2 Hz, H^{a}), 7.57 (1H, ddd, $^{3}J_{HTe}$ = 17.3 Hz, $^{2}J_{HH}$ = 6.9, 3.9 Hz, H^{b}), 7.34 (1H, m, H^{c}), 6.70 (2H, s, H^{l}), 5.80 (1H, br s, H^{h}), 3.78-3.58 (2H, m, H^{j}), 3.51-3.42 (2H, m, H^{i}), 3.20 (2H, dt, $^{3}J_{HTe}$ = 16.0 Hz, $^{3}J_{HH}$ = 7.1 Hz, H^{e}), 2.47 (2H, t, $^{3}J_{HH}$ = 7.1 Hz, H^{f}). $^{13}C\{^{1}H\}$ NMR ($CDCl_3$, 125 MHz): δC 172.16 (s, C^{g}), 171.03 (s, C^{k}), 149.24 (d, $^{1}J_{CTe}$ = 302.9 Hz, C^{d}), 136.97 (d, $^{2}J_{CTe}$ = 5.1 Hz, C^{b}), 135.66 (s, C^{c}), 134.35 (s, C^{l}), 125.04 (d, $^{1}J_{CTe}$ = 298.5 Hz, C^{a}), 39.90 (d, $^{3}J_{CTe}$ = 6.0 Hz, C^{f}), 39.18 (s, C^{i}), 37.61 (s, C^{j}), 32.37 (d, $^{2}J_{CTe}$ = 31.1 Hz, C^{e}).