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Multiplexed single-cell analysis of organoid signaling networks

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Organoids are biomimetic tissue models comprising multiple cell types and cell states. Post-translational modification (PTM) signaling networks control cellular phenotypes and are frequently dysregulated in diseases such as cancer. Although signaling 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 signaling 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 cytometry by time of flight (CyTOF) signaling analysis pipeline (CyGNAL) for computing cell type–specific PTM signaling networks. The TOB*is* MC protocol takes ~3 d 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-organizing 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 personalized cancer therapy[7–9]. Although incredibly powerful, organoids are heterogeneous model systems that are challenging to analyze by using conventional technologies.

Cells within an organoid can be classified with a 'cell type' (e.g., stem and differentiated) and a 'cell state' (e.g., proliferating and 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 (PTM) signaling networks[11]. Common PTMs include protein phosphorylation, methylation, acetylation and ubiquitination[12]. PTM signaling networks are frequently dysregulated in cancer, and PTM signaling nodes such as kinases are targeted by many anti-cancer drugs[13]. Because PTM signaling 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 signaling 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 use organoids in biomedical research, we must be able to quantify and compare multiple organoid PTM signaling 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, cell state and PTM signaling in small intestinal organoids and uncovered a novel connection between oncogenic

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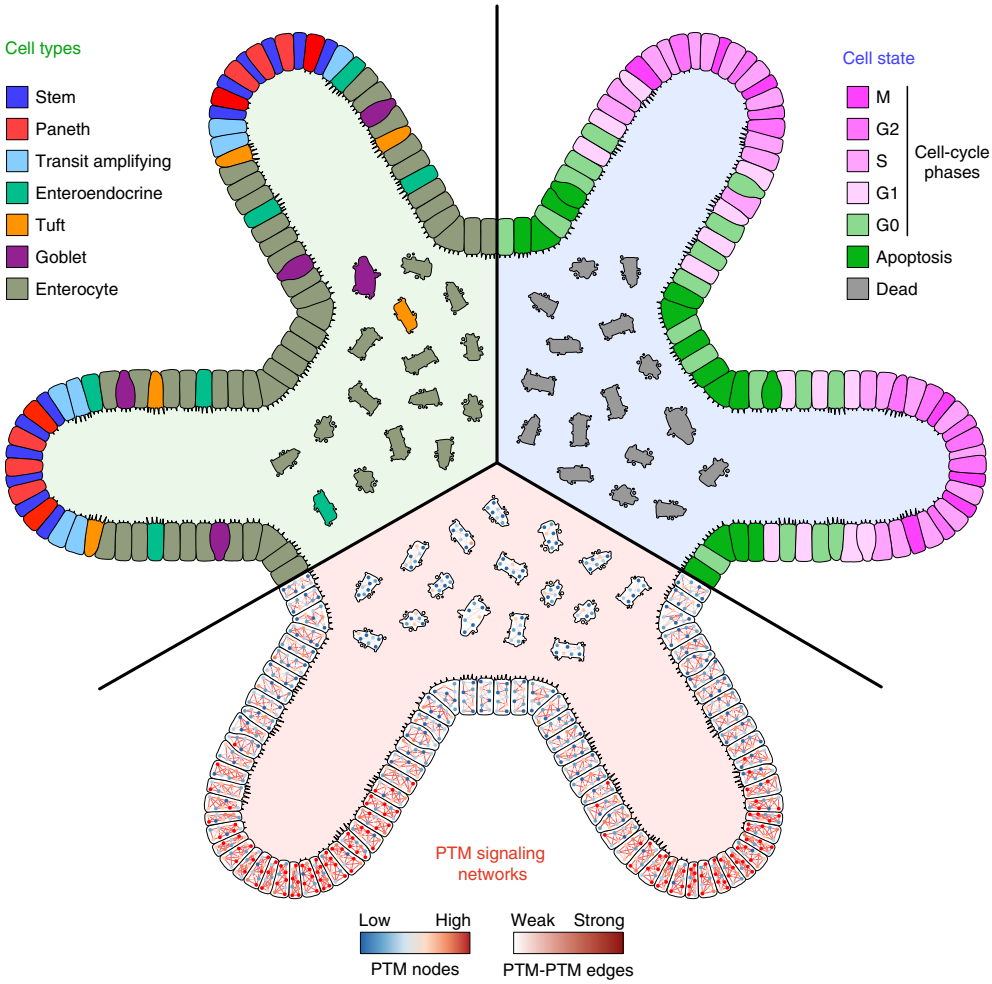


Fig. 1 | Organoids are high-dimensional systems. Schematic of a small intestinal organoid. Small intestinal organoids contain multiple cell types, each in a unique cell state. Each cell contains cell type– and cell state–specific post-translational modification signaling networks. High-dimensional technologies are needed to simultaneously quantify cell type– and cell state–specific PTM signaling in organoids.

and microenvironmental signaling cues in colorectal cancer (CRC) tumor 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 pretreatment (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 signaling in murine heterocellular organoids by using a 20-plex TOB*is* barcoding strategy (via a *6*-choose-*3* combination, i.e., 3 isotopes 'on' and 3 isotopes 'off')[17]. We have since 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 that the protocol is compatible with human PDOs and developed CyGNAL (CyTOF signaling analysis), a computational pipeline for analyzing high-dimensional PTM signaling MC data.

Comparison with other methods

Immunofluorescent technologies are limited by the spectral overlap of reporters, susceptible to autofluorescent artefacts and inherently low dimensional. Immunofluorescent imaging parameters

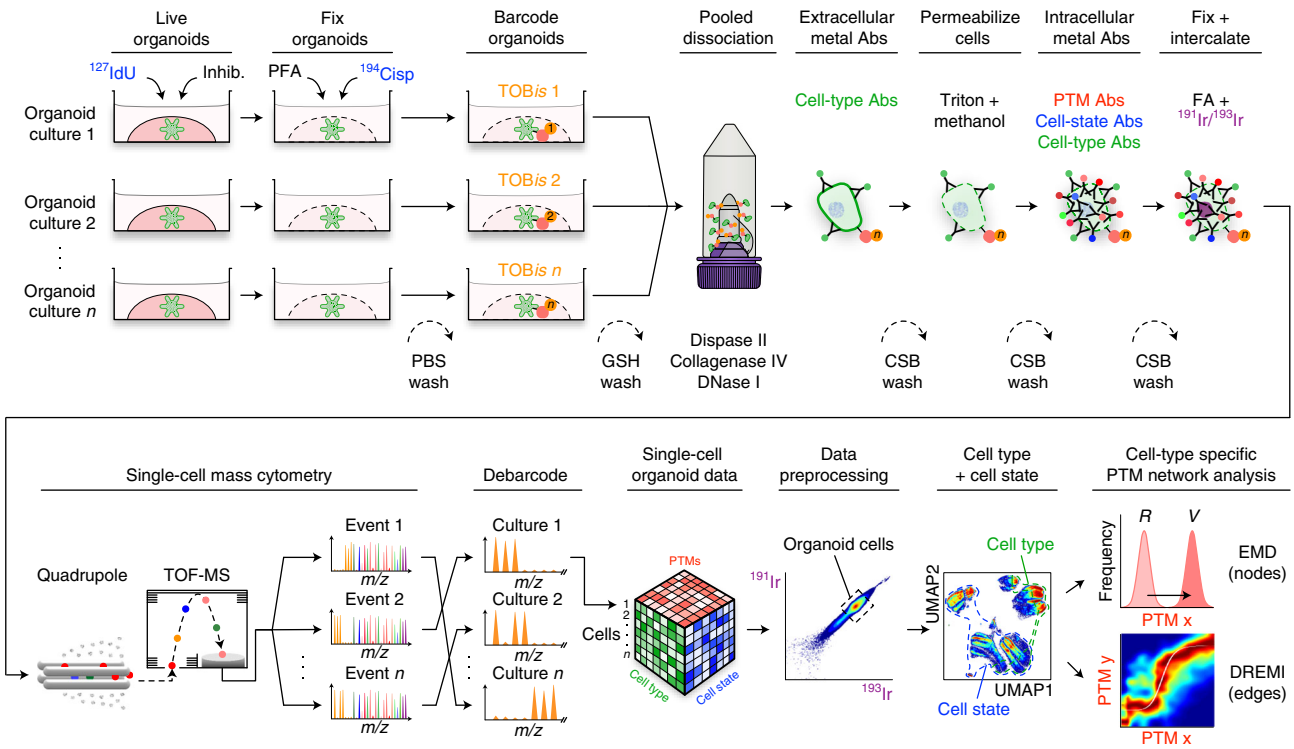


Fig. 2 | TOB*is* MC protocol overview. Live organoids are treated with ^{127}5-iodo-2′-deoxyuridine (identifying S-phase cells) and phosphatase and protease inhibitors (Procedure Steps 1–3). Organoids are then fixed in situ with paraformaldehyde and stained with ^{194}cisplatin (^{194}Cisp) to identify dead/dying cells (Steps 4–7). 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 8–10). Organoids are dissociated into single cells by using dispase II, collagenase IV and DNase I and stained with extracellular cell-type rare earth metal-conjugated antibodies (Abs) (Steps 11–24). Cells are then permeabilized with Triton and methanol, stained with intracellular PTM, cell-state and cell-type Abs (Steps 25–33). Abs are cross-linked to their antigens by using formaldehyde (FA), and cells are incubated with $^{191/193}$Iridium (Ir) DNA intercalators (Steps 34–37). Single cells are analyzed by using a mass cytometer (Steps 38–49). Different experimental conditions are debarcoded and preprocessed, and single-cell organoid data are visualized by using uniform manifold approximation and projection (UMAP) (Steps 50–60). Cell type–specific PTM node intensity is calculated by using earth mover's distance (EMD) between reference (*R*) and variable (*V*) populations, and PTM-PTM connectivity is calculated by using density resampled estimation of mutual information (DREMI) (Steps 61–70). CSB, cell staining buffer (see Reagents); *m/z*, mass-to-charge ratio; TOF-MS, time-of-flight mass spectrometer.

can be expanded through multiple cycles of staining and quenching[19], but this is challenging to implement on heterogeneous and delicate 3D organoid cultures. Although fluorescent flow cytometry parameters can be increased by 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 signaling data from organoid co-cultures. In contrast, TOB*is* MC provides cell type–specific cell-state quantification and PTM signaling networks for every cell in an organoid culture. The high-parameter capacity of MC is particularly suited to analyzing PTM signaling in organoids co-cultured with stromal fibroblasts and leukocytes[17].

High-dimensional phenotyping of heterocellular systems is commonly performed by using single-cell RNA-seq (scRNA-seq)[22,23]. Although a mature technology to identify transcriptionally regulated differentiation trajectories, scRNA-seq workflows to measure 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, because 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 toward healthy cells and not well suited to analyzing cell death in organoids (as might be common in a PDO drug screen[7]). By contrast, MC can analyze viable, stressed and dead cells and is therefore capable of assessing apoptotic mechanisms in perturbed organoid cultures.

Cell states and PTM signaling 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 paraformaldehyde (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 ~1–10 × 10^3 cells per run. Given that a typical 12-well plate organoid culture contains ~0.5–1 × 10^6 cells per well, droplet-based scRNA-seq methods can capture only ~0.1–1% of the cells in such an experiment. In comparison, TOB*is* MC routinely analyzes >1 × 10^6 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 d. 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

Because TOB*is* MC requires organoids to be dissociated into a single-cell suspension, all spatial information is lost. Methods such as imaging mass cytometry[31], multiplexed ion beam imaging[31] or in situ scRNA-seq[32] should be considered when high-dimensional spatial phenotyping is required. Although MC can in theory be used to measure any cell sample that can be dissociated into single cells, we have not optimized TOB*is* for tissue samples. We recommend methods specifically optimized for fixed tissue such as DISSECT[28] for measuring PTM signaling in tissue. MC cannot describe intercellular signaling 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. Although 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 signaling networks in organoids and organoids co-cultured with stromal and immune cells[17]. The protocol is also well suited to studying how organoid signaling networks can be regulated by stromal and immune cells, including cellular therapies such as chimeric antigen receptor T cells (C.J.T. and Callum Nattress, University College London Cancer Institute, unpublished observations, 2021). We have successfully applied TOB*is* MC to multiple genotypic and microenvironmental conditions[17]. Although the workflow was developed by 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 signaling networks in organoids cultured in a protein-rich extracellular matrix such as Matrigel. Organoids can be grown in 6-well (×7 40-µl droplets), 12-well (×3 30-µl droplets), 48-well (×1 30-µl droplet) and 96-well (×1 50-µl stack) culture

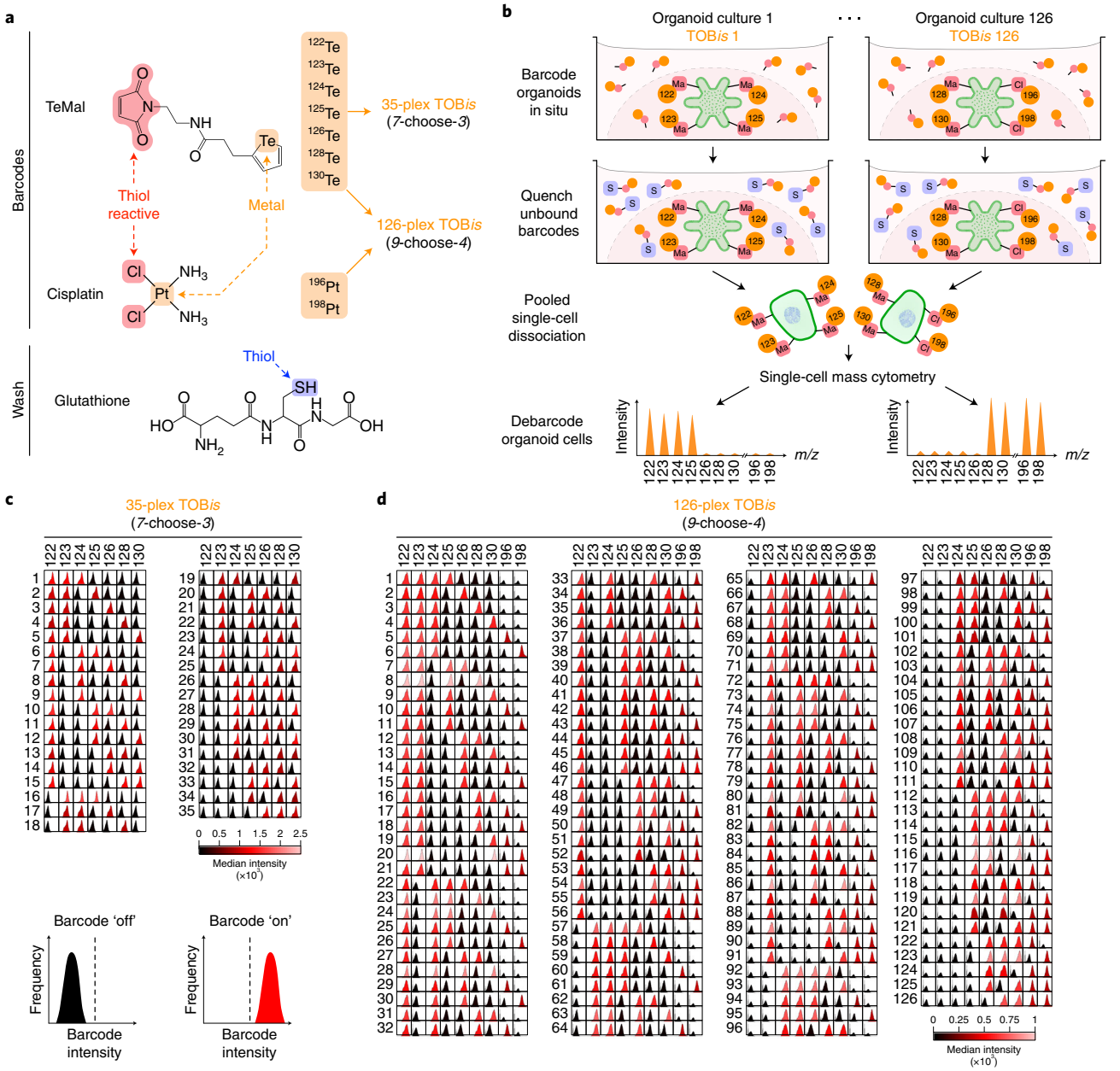
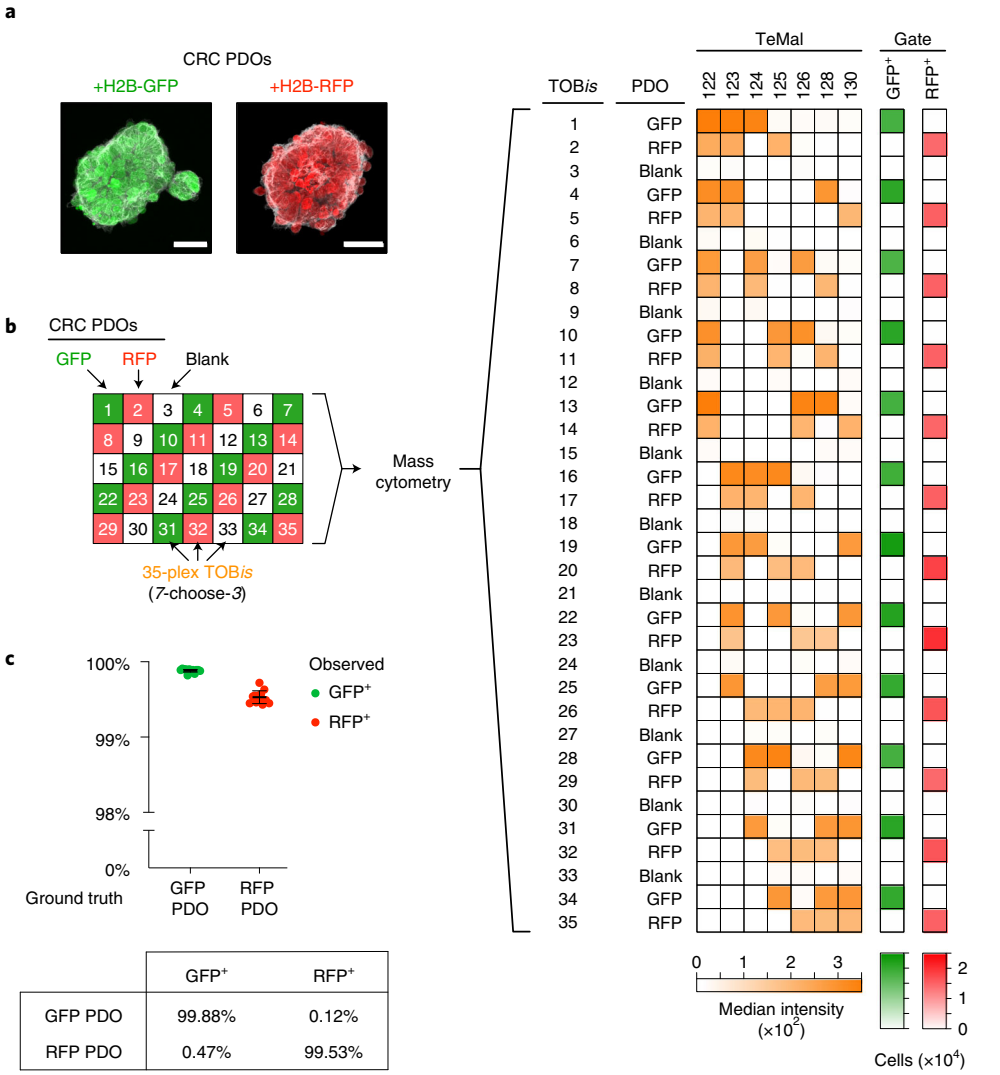


Fig. 3 | TOB*is* multiplexing overview. a, TOB*is* reagents. Seven thiol-reactive tellurium maleimide (TeMal) isotopologues (^{122}Te, ^{123}Te, ^{124}Te, ^{125}Te, ^{126}Te, ^{128}Te and ^{130}Te) are combined to form a doublet-filtering 35-plex (7-choose-3) barcoding matrix. Two additional cisplatin 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 (colored by the median intensity) of 35 murine *shApc/Kras*$^{G12D/+}$ CRC organoid cultures barcoded by 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, three of the seven Te isotopes need to be 'on', and the other four isotopes need to be 'off' to achieve successful sample demultiplexing. **d**, Histograms of monoisotopic Te and Pt (colored by the median intensity) of 126 murine *shApc/Kras*$^{G12D/+}$ CRC organoid cultures barcoded by 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, four of the nine Te/Pt isotopes need to be 'on', and the other five isotopes need to be 'off' to achieve successful sample demultiplexing.

formats. Because MC can measure both viable and apoptotic cells, the method can be used to analyze both newly seeded and fully developed organoids[17]. The current protocol is optimized 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



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

Fig. 4 | TOB*is* MC barcoding fidelity. a, Confocal microscopy of human CRC PDOs transfected with either H2B-GFP (endogenous, green) or H2B-RFP (endogenous, red) and 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 analyzed by MC. **c**, Percentage of GFP$^+$ and RFP$^+$ 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 s.d.

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 prefixation treatment

After organoid culture, ^{127}5-iodo-2′-deoxyuridine (^{127}IdU) is added to the media of live organoid cultures 30 min before the assay endpoint. ^{127}IdU integrates into the replicating genome of cells in S phase and can be easily monitored by MC[41]. 5 min before the 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 optimize the use of any such pretreatments 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 signaling profiles[28]. To avoid disruption of in situ cell states and PTMs in organoids, organoids are fixed while still in Matrigel. At the assay

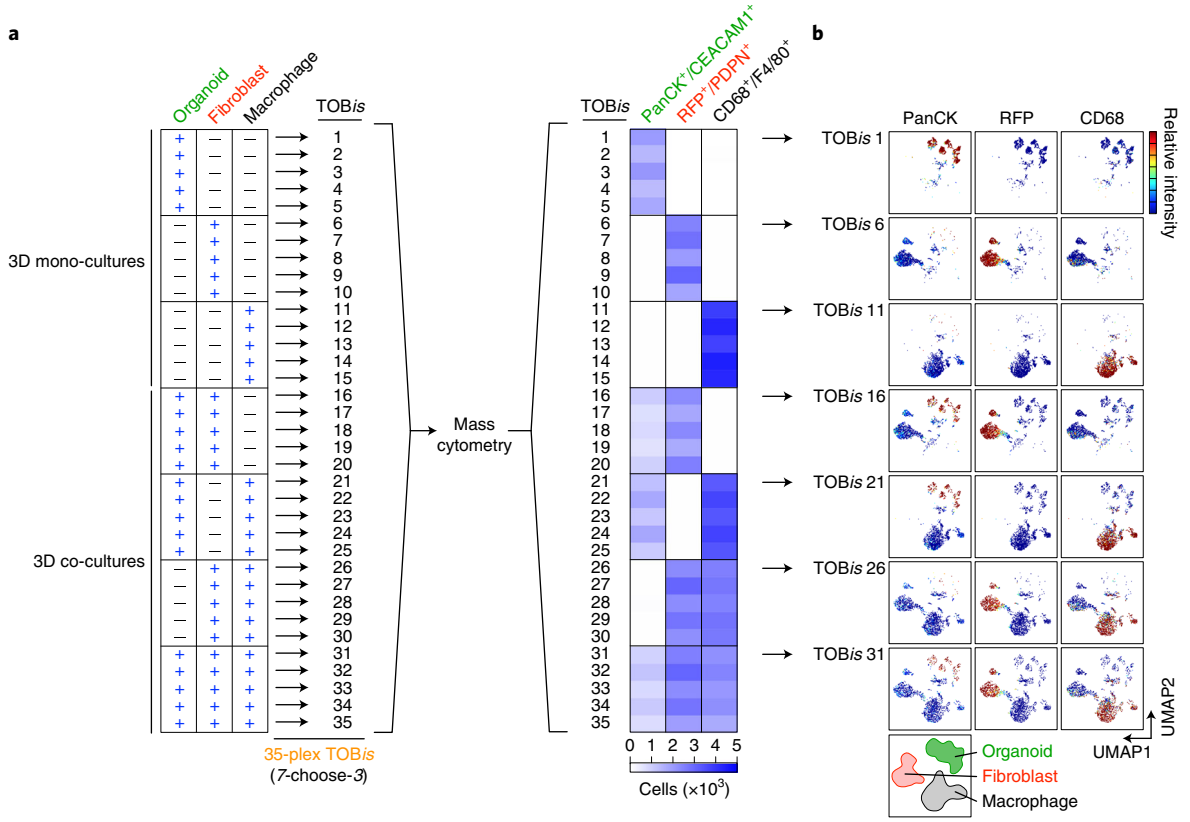


Fig. 5 | TOB*is* MC for organoid co-cultures. a, Mono- and co-cultures of organoids, fibroblasts and macrophages ($n = 5$) barcoded by using 35-plex (*7*-choose-*3*) TOB*is* and analyzed by MC. The heatmap shows numbers of organoids (pan-cytokeratin (PanCK)$^+$ and CEACAM1$^+$), fibroblasts (podoplanin (PDPN)$^+$ and RFP$^+$) and macrophages (CD68$^+$ and F4/80$^+$) recovered from each TOB*is* barcode. TOB*is* MC specifically resolved all the conditions and successfully labeled 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.

endpoint, culture medium is removed and replaced with 4% (wt/vol) PFA and incubated at 37 °C for 60 min. Fixed organoids are then washed with PBS and stained with monoisotopic cisplatin (e.g., ^{194}cisplatin or ^{198}cisplatin) for 10–15 min (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].

TOB*is* multiplexing

Mass-tagged cellular barcoding 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 mass-tagged cellular barcoding reagents (commonly used for barcoding leukocytes in suspension) are not suitable for labeling organoids while embedded in Matrigel[17]. To overcome this, we developed 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' and 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).

We premix TeMals and cisplatins into TOB*is* barcodes either by hand or by using an Opentrons OT-2 robot (https://github.com/TAPE-Lab/OT-2-Automated-Barcode-Pipetting) and store 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 °C. The following day, unbound thiol-reactive barcodes are quenched by using reduced glutathione 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

After 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 optimization, we found that dispase II breaks cell–cell contacts, collagenase IV digests Matrigel 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 optimized 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 by using a gentleMACS Octo dissociator. After dissociation, single organoid cells are washed and filtered to remove clumps. Cells are then stained for extracellular epitopes with rare earth metal-labeled antibodies (hereafter referred to as metal antibodies). Preconjugated metal antibodies can be purchased via Fluidigm or custom conjugated by using established protocols[47]. Cells are then permeabilized by using 0.1% (vol/vol) Triton X-100, 50% (vol/vol) methanol or both. Permeabilization buffers can greatly alter antibody staining and should be optimized for each biological system and antibody panel (Supplementary Fig. 5). Once permeabilized, 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 by using 1.6% (wt/vol) formaldehyde (FA). Finally, cells are incubated in DNA intercalator $^{191/193}$Ir overnight before 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 0.8–1.2 × 10^6 cells/ml and spiked with isotopic EQ beads[48]. Cells are then loaded into a Super Sampler (Victorian Airships), and single-cell data are acquired by using a mass cytometer (e.g., Fluidigm Helios). During optimization, we found that adding 2 mM EDTA to the running buffer and using the Super Sampler greatly improve the acquisition of epithelial organoid cells by MC (J.S. and X.Q., unpublished observation, 2018).

TOB*is* MC single-cell data analysis

After data acquisition, raw MC data are normalized[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 (https://github.com/TAPE-Lab/CyGNAL)[49] (Supplementary Fig. 6). The components of CyGNAL were previously used to analyze the datasets described by Qin et al.[17]. In brief, the preprocessing 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., uniform manifold approximation and projection (UMAP)[50]) can be performed on cell-comprised datasets and is mainly used as a visualization 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 visualized with heatmaps and further summarized by 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 signaling of heterocellular organoids[17].

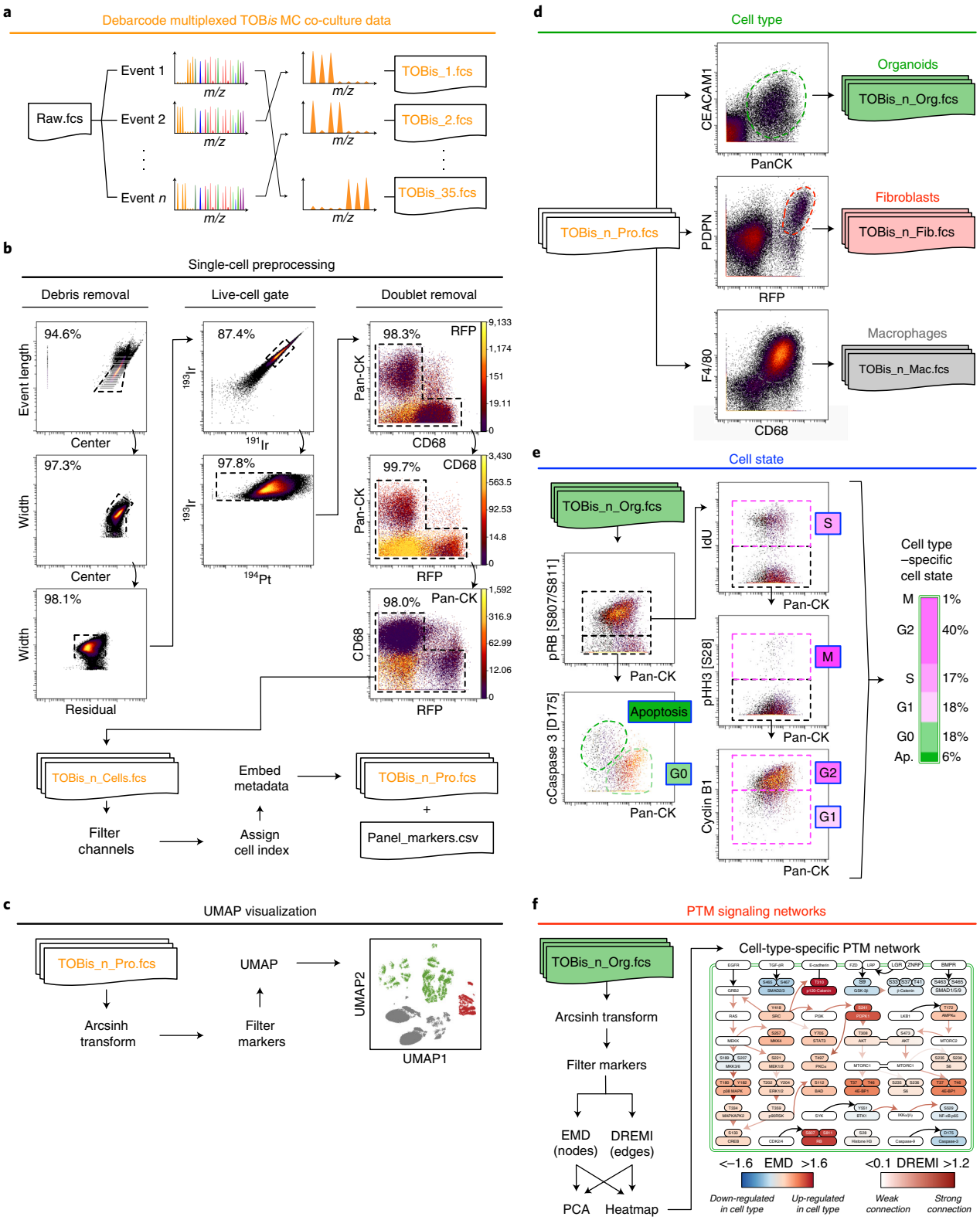


Fig. 6 | TOB*is* MC data analysis using CyGNAL. a, TOB*is* MC raw data are debarcoded[44] into individual experimental conditions (TOBis_n.fcs). **b**, All debarcoded data files are imported into a cytometry data analysis platform for debris removal, live-cell identification and doublet removal (percentages 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 preprocessed 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 by using user-defined markers. **d**, Cell-type identification is performed on preprocessed data to generate cell type–specific datasets. **e**, Cell-state analysis is performed on cell type–specific data. **f**, PTM signaling analysis is performed on cell type–specific data via EMD[51,52] and DREMI[53] calculations. EMD and DREMI scores can be visualized in heatmaps or summarized by using PCA.

Materials

Biological materials

- Wild-type, Apc knockdown (*shApc*) and *shApc/Kras*$^{G12D/+}$ murine colon organoids[54] (gift from L. Dow, Cornell University)
- Wild-type murine small intestinal organoids (gift from V. Li, Crick Institute)
- CRC PDOs[7] (gift from M. Garnett, Sanger Institute)
- Immortalized wild-type colonic fibroblasts[17]
- Bone marrow–derived macrophages[17]

Reagents

Organoid culture

- Growth factor reduced Matrigel (Corning, cat. no. 354230)
- Advanced DMEM/F-12 (Thermo Fisher Scientific, cat. no. 12634010)
- L-Glutamine (Thermo Fisher Scientific, cat. no. 25030081)
- *N*-Acetyl-L-Cysteine (Sigma, cat. no. A9165)
- HEPES (Sigma, cat. no. H3375)
- B-27 supplement (Thermo Fisher Scientific, cat. no. 17504044)
- N-2 supplement (Thermo Fisher Scientific, cat. no. 17502048)
- HyClone penicillin-streptomycin solution (Thermo Fisher Scientific, cat. no. SV30010)
- Murine epidermal growth factor (mEGF) (Thermo Fisher Scientific, cat. no. PMG8041)
- Murine noggin (Peprotech, cat. no. 250-38)
- Murine R-spondin-1 (Peprotech, cat. no. 315-32)
- Murine Wnt-3a (Peprotech, cat. no. 315-20)
- Human R-spondin-1 (Peprotech, cat. no. 120-38)
- Gastrin I (Sigma, cat. no. SCP0152)
- A83-01 (Generon, cat. no. 04-0014)
- SB202190 (Cayman Chemical, cat. no. 10010399)
- Nicotinamide (Merck, cat. no. N0636)

Mass cytometry

- ^{127}IdU (Fluidigm, cat. no. 201127)
- Protease inhibitor cocktail(Sigma, cat. no. P8340)
- PhosSTOP (Sigma, cat. no. 4906845001)
- PFA solution, 4% (vol/vol) in PBS (Thermo Scientific, cat. no. J19943K2) **! CAUTION** PFA is a mutagenic and carcinogenic agent. Avoid eye or skin contact.
- ^{194}Cisplatin (Fluidigm, cat. no. 201194)
- ^{196}Cisplatin (custom order from BuyIsotope)
- ^{198}Cisplatin (Fluidigm, cat. no. 201198) **! CAUTION** Cisplatin is mutagenic and carcinogenic. Avoid eye or skin contact.
- L-Glutathione (Sigma, cat. no. G6529)
- Dispase II (Thermo Fisher Scientific, cat. no. 17105041)
- Collagenase IV (Thermo Fisher Scientific, cat. no. 17104019)
- DNase I (Sigma, cat. no. DN25)
- TeMal (^{122}Te, ^{123}Te, ^{124}Te, ^{125}Te, ^{126}Te, ^{128}Te and ^{130}Te) (see Supplementary Method)
- Metal antibodies (various suppliers; see Supplementary Table 2 for an example antibody panel)
- Maxpar cell staining buffer (CSB; Fluidigm, cat. no. 201068)
- Maxpar X8 metal labeling kit (Fluidigm, cat. no. 201300)
- Maxpar water (Fluidigm, cat. no. 201069)
- Maxpar PBS (Fluidigm, cat. no. 201058)
- Maxpar Fix and Perm buffer (Fluidigm, cat. no. 201067)
- EDTA (Sigma, cat. no. 03690-100ML)
- Triton X-100 (Sigma, cat. no. T8787)
- Methanol (Fisher, cat. no. 10675112)
- Pierce 16% (wt/vol) FA, methanol free (Pierce, cat. no. 28906)
- Cell-ID Intercalator-Ir (Fluidigm, cat. no. 201192A)
- EQ four element calibration beads (Fluidigm, cat. no. 201078)

Reagent setup

Murine colonic organoid monoculture and co-culture medium

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 murine noggin, 500 ng/mL murine R-spondin-1, 10 mM nicotinamide and 1× HyClone penicillin streptomycin solution. ▲**CRITICAL** 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 on the basis of their model system.

CRC PDO culture medium

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 murine noggin, 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. ▲**CRITICAL** 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 on the basis of their model system.

Heavy metal–conjugated antibodies

Metal antibodies can be purchased preconjugated from Fluidigm or custom conjugated with monoisotopic heavy metals purchased from Fluidigm or Trace Sciences by 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 (using 1 μg of antibody/ml of CSB as a starting point) 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 signaling[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) and 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 more than two 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.

TOB*is*

Debarcoding efficiency is heavily dependent on robust signal intensities of the Te and Pt channels. Because barcode signal intensities can vary between isotopologues, barcode batch and cell types being labeled, we advise titrating TeMal and cisplatin barcodes to achieve an 'on' median intensity $>5 \times 10^2$ and an 'off' median intensity $<1 \times 10^2$ 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 and ^{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 divide them into aliquots 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 by using a liquid-handling robot. Scripts to prepare both 35-plex (*7*-choose-*3*) and 126-plex (*9*-choose-*4*) TOB*is* barcodes by using the OT-2 platform (Opentrons) are provided at https://github.com/TAPE-Lab/OT-2-Automated-Barcode-Pipetting. Premixed TOB*is* barcodes can be kept at −80 °C for long-term storage before use. Although this protocol is designed for TeMal and cisplatin reagents, alternative thiol-reactive heavy metal probes (e.g., lanthanide-conjugated mDOTA (maleimide dodecane tetraacetic acid)[17]) could also, in theory, be used to perform TOB*is*.

Equipment

- gentleMACS C-Tube (Miltenyi, cat. no. 130-096-334)
- gentleMACS Octo dissociator (with heaters) (Miltenyi, cat. no. 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 two custom programs listed below were designed for the gentleMACS Octo dissociator.

Standard protocol custom program.
- Set heater temperature to 37 °C
- Forward-spin at 20 rpm for 2 min
- Backward-spin at 20 rpm for 2 min
- Loop 15×:
  - Forward-spin at 1,500 rpm for 2 s
  - Backward-spin at 1,500 rpm for 2 s
  - Forward-spin at 50 rpm for 3 min

Quick protocol custom program.
- Set heater temperature to 37 °C
- Forward-spin at 50 rpm for 1 min
- Backward-spin at 50 rpm for 1 min
- Loop 10×:
  - Forward-spin at 1,500 rpm for 2 s
  - Backward-spin at 1,500 rpm for 2 s
  - 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 are >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, the oxide ratio (M1/M2) displayed in gases should be <0.03.
- The percent 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*, *rpy2*, *scprep*, *sklearn* and *umap-learn*
  - R >3.6 (https://www.r-project.org/) with libraries *ComplexHeatmap*, *DT*, *factoextra*, *FactoMineR*, *flowCore*, *Ggally*, *ggrepel*, *ggplot2*, *Hmisc*, *MASS*, *matrixStats*, *plotly*, *psych*, *RColorBrewer*, *shiny* and *tidyverse*
- MATLAB (https://www.mathworks.com/products/matlab.html)
- Single Cell Debarcoder (https://github.com/zunderlab/single-cell-debarcoder)

Procedure

▲ **CRITICAL** TOB*is* MC can be used to assess organoids cultured in 6-, 12-, 48- or 96-well formats. This protocol describes how to analyze 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 optimized organoid culture conditions as inputs for TOB*is* MC.

Culture organoids

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

▲ **CRITICAL STEP** For the results illustrated in this paper, we have cultured murine intestinal organoids (Fig. 3), human CRC PDOs (Fig. 4) and murine organoids co-cultured with colonic fibroblasts and/or macrophages (Fig. 5) for 3 d in 50 µl of Matrigel and 200 µl of medium. Users should use optimized culture conditions relevant to their own organoids and biological questions as inputs for TOB*is* MC.

S phase cell labeling ● Timing ~30 min

2 Add ^{127}IdU directly to culture medium to a final concentration of 25 µM (10 µl of 0.5 mM stock added to 200 µl of medium). Gently rotate the plate by hand five times for 10 s to mix the medium and incubate the plate for 25 min at 37 °C and 5% CO_2.

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

Phosphatase and 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 medium, gently rotate the plate by hand five times for 10 s and incubate for 5 min at 37 °C and 5% CO_2.

▲ **CRITICAL STEP** Protease and phosphatase inhibitors have been shown to help preserve cell signaling and antigen stability during fixation[28]. However, because 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 medium by pipetting. Add 200 µl of prewarmed (37 °C) 4% (vol/vol) PFA into each well, taking care not to disrupt Matrigel. Incubate for 60 min at 37 °C and 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 prewarm PFA to 37 °C.

▲ **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 °C). Repeat the wash.

■ **PAUSE POINT** Fixed cells can be kept at 4 °C 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** Because dead cells can be found inside organoid structures (Fig. 1), it is crucial that organoids be 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 that 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 the 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 analyzed, skip to Step 11.

TOB*is* (optional) ● Timing ~30 min of bench work; incubation overnight

8 Transfer 200 µl of 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 °C.

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

▲ **CRITICAL STEP** Ensure that 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 high-density cultures are needed.

9 Remove the barcoding solutions by pipetting and wash the cells with 200 µl of 2 mM glutathione/CSB for 10 min on a rocker at room temperature. Repeat the 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 the wash.

■ **PAUSE POINT** The barcoded cells can be kept at 4 °C for ≤4 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 on the basis of 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 minimize 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 that all cells are recovered.

14 Dissociate organoids into single cells by using a gentleMACS Octo dissociator and 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 the supernatant.

18 Wash cells with 2 ml of CSB, centrifuge at 800*g* for 5 min at room temperature and discard the supernatant. Repeat the wash.

19 Resuspend cells in 2 ml of 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 on the basis of the cellular composition of their experimental system.

20 Count cells by using the Countess II automated cell counter. Up to ~4.5×10^6 cells can be taken forward for 1× MC staining.

■ **PAUSE POINT** The fixed, barcoded and dissociated cells can be kept at 4 °C for ≤4 weeks in CSB.

? TROUBLESHOOTING

Extracellular stain ● Timing ~45 min

21 Centrifuge cells at 800*g* for 5 min at room temperature, discard the supernatant and resuspend cells in 50 µl of CSB.

22 Prepare extracellular antibody cocktail by mixing the antibody panel (see Supplementary Table 2 for an example) 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 min to avoid cells pelleting under gravity.

24 Add 2 ml of CSB to the cells, centrifuge at 800*g* for 5 min at room temperature and discard the supernatant.

Permeabilization ● Timing ~45 min

25 Resuspend cells in 1 ml of 0.1% (vol/vol) 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 min to avoid cells pelleting.

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

27 Place the cells on ice for 1 min.

28 Resuspend cells in 1 ml of ice-cold 50% (vol/vol) methanol/PBS (store at −20 °C until use), gently vortex and incubate for 10 min on ice.
▲ **CRITICAL STEP** Different permeabilization buffers can substantially alter antibody staining (Supplementary Fig. 5). Although we commonly use 0.1% (vol/vol) Triton X-100 followed by 50% (vol/vol) methanol, we advise users to optimize the permeabilization conditions that best suit their model system and antibody panel.

29 Add 2 ml of CSB to the cells, centrifuge at 800*g* for 5 min at room temperature and discard the supernatant. Repeat the wash.

30 Resuspend cells in 50 µl of CSB.

Intracellular stain ● Timing ~45 min

31 Prepare intracellular antibody cocktail by mixing the antibody panel (see Supplementary Table 2 for an example) 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 min to avoid cells pelleting.

33 Add 2 ml of CSB to the cells, centrifuge at 800*g* for 5 min at room temperature and discard the supernatant.

Post-staining fixation ● Timing ~15 min

34 Add 1 ml of 1.6% (vol/vol) FA/PBS solution made fresh from 16% (vol/vol) FA to the cells and incubate for 10 min on a rocker at room temperature.
▲ **CRITICAL STEP** The post-staining fixation step is required if the sample needs to be stored for >48 h before data acquisition.

35 Add 2 ml of CSB to the cells, centrifuge at 800*g* for 5 min at room temperature and discard the supernatant.

DNA intercalation ● Timing ~1 h/overnight

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

37 Resuspend cells in 1 ml of intercalation buffer, gently vortex and incubate for 1 h on a rocker at room temperature or overnight at 4 °C.
▲ **CRITICAL STEP** Because the intercalation reaction is non-covalent, cells should be kept at 4 °C 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 °C in the intercalation buffer for ≤2 weeks (with post-staining fixation) or 48 h (without post-staining fixation).

MC data acquisition ● Timing ≥1 h (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 h), 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 the supernatant.

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

42 Wash cells with 2 ml of MaxPar water, centrifuge at 800*g* for 5 min at room temperature and discard the 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 by using a 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 and 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 by using the Fluidigm CyTOF software. Aim for 100–400 events/s.

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

Debarcoding the TOB*is* multiplexed MC dataset ● Timing ~15 min

50 Debarcode multiplexed FCS file(s) (Fig. 6a, Raw.fcs) into separate experimental conditions by 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 Tables 3 and 4).
? 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 the 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 preprocessing ● 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 that 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 preprocess dataset(s) by 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 preprocessed 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 and Supplementary Fig. 6).

▲ **CRITICAL STEP** Preprocessing 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 (~1 million 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 labeling markers used for UMAP calculation with 'Y' (by default, all markers are labeled 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 and 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 on the basis of 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 visualization 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) on the basis of cell type–specific markers (Fig. 6d).

▲ **CRITICAL STEP** To improve the fidelity of cell-type identification, at least two 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 signaling network analysis.

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

PTM signaling 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 labeling markers used for the calculation with 'Y' (by default, all markers are labeled 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 visualized by using heatmaps (Steps 67 and 68) or summarized with PCA (Steps 69 and 70).

▲ **CRITICAL STEP** For EMD calculations, the user needs to define the reference (*R*) dataset for all experimental variables (*V*) against which to compare. 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* to indicate the 'direction' of signaling 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 visualization ● Timing <5 min

67 To visualize EMD/DREMI scores by 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 visualization 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 visualization 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 principal component can be exported separately as a TXT file.

Troubleshooting

Troubleshooting advice can be found in Table 1.

Table 1 | Troubleshooting table

Step	Problem	Possible reason	Solution
4	Matrigel droplets dissolve in PFA	The PFA solution is not warm enough	Prewarm 4% (vol/vol) PFA solution to 37 °C before 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 by using our custom dissociation program. We recommend that users prepare fresh dissociation solution before each use. Run additional rounds of the 'Quick Protocol' if needed

Table continued

Table 1 (continued)

Step	Problem	Possible reason	Solution
20	Considerable cell loss, especially after being transferred to a new FACS tube	Cell loss is inevitable during staining because of 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 that users start with >1 × 10^6 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 optimization, we observed that coating polypropylene FACS tubes with CSB before centrifugation of cells resuspended in PBS also facilitates the cells spinnig down properly and thereby increases cell recovery
49	Antibody staining is not working	The 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 permeabilization conditions for their specific experimental system
50	Experimental conditions are not debarcoded efficiently	Incorrect amounts of barcodes are added to the cells	Ensure that barcodes are accurately divided into aliquots and that all barcodes from each TOB*is* condition are added to the cell cultures
		The MC run acquires heavy metal contaminants accumulated during the staining steps, causing a lower-than-expected percentage of 'real' events	Adding 2 mM EDTA to the CSB wash buffer before MC data acquisition helps chelate free metals in the cell suspension and clean up the MC run
		An incompatible barcode key is provided to the Debarcoder	Make sure that 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	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) per their specific instructions
			Errors in R installation are probably 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
			Refer to the GitHub issue page for additional help

Timing

Steps 2–5, Pretreatment and fixation of organoids: ~2 h
Steps 6 and 7, live/dead discrimination staining: ~0.5 h
Step 8, TOB*is* barcoding: 1–2 h at room temperature or overnight at 4 °C
Steps 9 and 10, quenching of TOB*is* barcodes: ~0.5 h
Steps 11–20, single-cell dissociation: 1–2 h
Steps 21–35, MC staining: ~2.5 h
Steps 36 and 37, DNA intercalation: 1 h at room temperature or overnight at 4 °C
Steps 38–49, data acquisition: 1–2 h, depending on the number of cells to be analyzed
Steps 50–70, data analysis: 2–4 h, depending on the scale of the experiment

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% to 70% of the total debarcoded event count. Previous analysis with small intestinal organoids confirmed that 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 preprocessing, 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 visualized by using UMAP[50] via CyGNAL (or *t*-SNE (*t*-distributed stochastic neighbor embedding)[60]/PHATE (potential of heat diffusion for affinity-based trajectory embedding)[61] by 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 visualized by 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 signaling networks between organoids and organoid co-cultures.

Reporting Summary

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

Data availability

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

Code availability

The latest CyGNAL pipeline is available at https://github.com/TAPE-Lab/CyGNAL. CyGNAL version 0.2.1 as described in this publication can be found at https://github.com/TAPE-Lab/CyGNAL/releases/tag/v0.2.1. The OT-2 barcode preparation code is available at https://github.com/TAPE-Lab/OT-2-Automated-Barcode-Pipetting. The code in this paper has been peer reviewed.

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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, analyzed the data and wrote the manuscript. F.C.R. developed CyGNAL and wrote the manuscript. P.V. and M.R.Z. performed organoid and MC experiments. Y.J.B. and M.N. developed TeMal reagents. C.J.T. designed the study, analyzed the data and wrote the manuscript.

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.

Additional information

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41596-021-00603-4.

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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection: Mass cytometry data in this study was collected with a Helios mass cytometer using the Fluidigm CyTOF Software (Version 6.7).

Data analysis: Data analysis was performed with Cytobank (Version 7.2.0) and publicly available R and python packages. Specifically:

Python >3.6 with packages:
fcsparser
fcswrite
numpy
pandas
plotly
rpy2
scprep
sklearn
umap-learn

R >3.6 with packages:
DT
factoextra
FactoMineR
flowCore
Ggally
ggplot2
Hmisc

MASS
matrixStats
plotly
psych
RColorBrewer
shiny
tidyverse

MATLAB (R2020b):
Single Cell Debarcoder (https://github.com/zunderlab/single-cell-debarcoder)

Statistical tests were performed using Graphpad Prism (Version 7.0).
Immunofluorescence staining images were processed using FIJI (Version 2.0.0).

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Data

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All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	No sample size calculation was performed as this is a method development and proof-of-concept project. Sample size was determined based on the experience and expertise of the investigators.
Data exclusions	All cells were gated for Gaussian discrimination parameters (Event length, Centre, Residual, and Width values) as recommended by Fluidigm to remove non-events such as debris and doublets. The cell-gating strategy was described in Figure 6.
Replication	Multiple cohorts of murine and patient-derive organoids were used in this project, yielding comparable results and thereby validated the robustness of our methods. Details on technical/biological replications of experiments are included in relevant figure legends.
Randomization	Animals and organoid samples were randomly chosen for inclusion in this study.
Blinding	No conditions presented in this study required blinding.

Reporting for specific materials, systems and methods

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☐	☒ Antibodies
☐	☒ Eukaryotic cell lines
☒	☐ Palaeontology and archaeology
☐	☒ Animals and other organisms
☒	☐ Human research participants
☒	☐ Clinical data
☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Antibodies

Antibodies used

All antibody information, including clone name and supplier, is provided in Supplementary Table 2 with the addition of the following antibodies:
CEACAM1, Clone CC1, Thermo Fisher
Podoplanin, Clone 8.1.1, BioLegend
RFP, Clone 8E5.G7, 2BScientific
F4/80, Clone BM8, BioLegend
CD68, Clone FA-11, BioLegend

Validation

Cell-type identification antibodies are validated in our previous publication Qin, X., Sufi, J., Vlckova, P. et al. Cell-type-specific signaling networks in heterocellular organoids. Nat Methods 17, 335–342 (2020). https://doi.org/10.1038/s41592-020-0737-8.
PTM antibodies selected for this study are widely used by the mass cytometry community and well-validated.
Antibody panels were carefully designed and titrated in accordance with known monoisotopic impurities and antigen abundance to ensure minimal cross-channel contamination.

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)

- Wild-type, Apc knockdown (shApc), and shApc / KrasG12D/+ murine colon organoids were a kind gift from Prof. Lukas Dow (Cornell University).
- Colorectal cancer patient-derived organoids were a kind gift from Dr. Mathew Garnett (Sanger Institute).
- Wild-type murine small intestinal organoids were a kind gift from Dr. Vivian Li (Crick Institute).
- Murine colonic fibroblasts were isolated and immortalised at UCL Cancer Institute as described in Qin, X., Sufi, J., Vlckova, P. et al. Cell-type-specific signaling networks in heterocellular organoids. Nat Methods 17, 335–342 (2020). https://doi.org/10.1038/s41592-020-0737-8.

Authentication

Cell lines used in this study have not been authenticated during the development of the project.

Mycoplasma contamination

Cells were checked for mycoplasma infection monthly using the MycoAlertTM PLUS Mycoplasma Detection Kit (Lonza LT07-701) and remained negative throughout this project.

Commonly misidentified lines
(See ICLAC register)

No commonly misidentified cell lines were used in this study.

Animals and other organisms

Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Laboratory animals

- Murine bone marrow-derived macrophages were isolated from 10- to 12-week-old C57BL/6 mice provided by Dr. Sophie Acton (University College London).

Wild animals

This study did not involve the use of wild animals.

Field-collected samples

This study did not involve the use of field-collected samples.

Ethics oversight

All animal work carried out was approved by local ethical review and licensed by the UK Home Office.

Note that full information on the approval of the study protocol must also be provided in the manuscript.