

RESEARCH LETTER

A Method for Cryogenic Preservation of Human Biopsy Specimens and Subsequent Organoid Culture



Human tissue-derived gastrointestinal (GI) organoids have revolutionized the study of human biology,¹⁻³ and are powerful tools for studying human physiology and disease; however, generation of organoids is limited by access to human tissue and a short window of viability for human samples, putting a hard limit on the time and place in which a patient sample can be used for research.⁴ These restraints mean that a laboratory must be relatively

geographically close to the source of collection to use the sample within the window of viability. Patient-derived organoids also are being used for drug development, stem cell therapies, and personalized medicine; however, it is not always feasible to prospectively develop organoid line efforts given the time and labor involved.^{5,6}

To overcome these limitations, we sought to develop a practical method to cryopreserve live human biopsy tissue, which then could be stored or shipped frozen and later thawed to generate new cultures of GI epithelium-only organoids (also referred to as enteroids/colonoids). Here, we describe a simple and robust method to cryopreserve human biopsy specimens that subsequently could be

thawed and used to generate epithelium-only organoids.

Results/Discussion

Endoscopic biopsy specimens were collected with 8-mm² biopsy forceps (average biopsy size, 5 mm²), transported to the laboratory, divided further into 2- to 3-mm² fragments, then transferred into a cryopreservation vial containing freezing medium. We tested 2 freezing media formulations: a complex medium (LWRN, fetal bovine serum, dimethyl sulfoxide, Y27632, CHIR99021; see [Supplementary Materials and Methods](#) section)^{7,9}; and a simple freezing medium (Dulbecco's modified Eagle medium/F12, 10% fetal bovine serum,

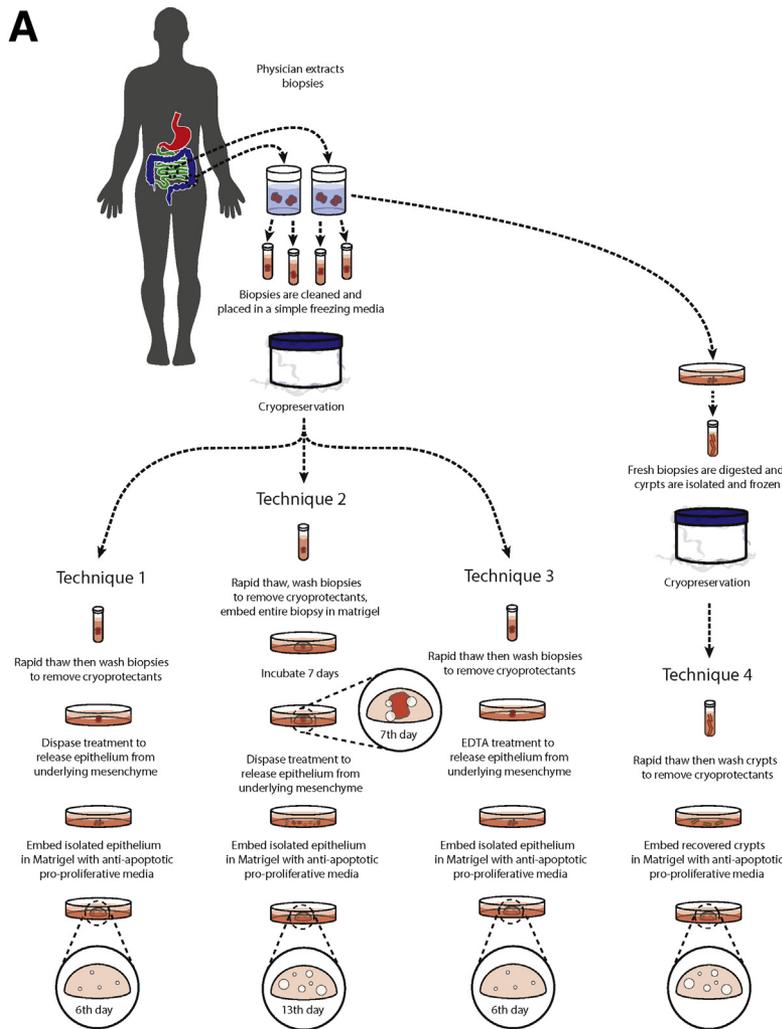


Figure 1. Schematic of the methods developed that allow successful generation of patient biopsy-derived epithelial organoids after cryopreservation. (A) The process of endoscopic biopsy collection followed by cryopreservation in a simple freezing medium can be accomplished in typical clinical settings with readily available equipment. After cryopreservation, organoid cultures can be established using 3 different techniques. Technique 1 (left) used a dispase digestion to isolate crypts from freshly thawed tissue. Technique 2 (middle-left) adds a step relative to technique 1 in which the entire biopsy specimen is embedded in Matrigel and allowed to repair from the freezing process before tissue digestion and crypt isolation. Technique 3 (middle-right) is very similar to technique 1 but uses a gentle EDTA treatment to separate the epithelium from the mesenchyme. Technique 4 (right) involves isolation of epithelial crypts before cryopreservation so that, upon thawing, cultures can be seeded immediately without additional tissue manipulations. All 4 techniques result in pure organoid cultures 2 weeks after initially thawing the biopsy specimen. (B) Organoids lines were generated using techniques 1 and 2. (C) Organoid lines were generated using technique 3. (D) Organoid lines were generated using technique 4.

B

Organoid lines generated using Technique 1 & 2

Patient ID	Patient Age (years)	Patient Sex	GI Region	Successful Enteroids (Fresh)	Successful Enteroids (Frozen)	Days Frozen at -80°C
98	70	Female	colon	yes	yes	3
99	59	Female	colon	yes	yes	3
100	67	Male	colon	yes	yes	3
101	59	Female	colon/ileum	yes/yes	yes/yes	3 & 10
102	61	Female	colon	yes	yes	3 & 10 & 48
103	63	Female	colon/ileum	yes/yes	yes/yes	3 & 10
104*	51	Male	colon/ileum	yes/yes	yes/yes	3
105*	19	Male	colon/ileum	yes/yes	yes/yes	3 & 34
114	43	Male	colon/duodenum	yes/yes	yes/yes	3
115	46	Female	duodenum	yes	yes	3
116	54	Female	duodenum	yes	yes	3
117	67	Female	duodenum	yes	yes	3
105	19	Male	colon	yes	yes	49 in LN2
127	52	Female	stomach	yes	yes	3
128	60	Female	stomach	yes	yes	3
129	60	Male	stomach	yes	yes	3
130	68	Male	stomach	yes	yes	3
130 [†]	58	Female	colon adenoma	yes	yes	3
132 [‡]	2	Male	ileum/sigmoid/cecum	N/A	yes/yes/yes	5 / 7 / 9 [§]
133 [‡]	unknown (adult)	Male	ileum/sigmoid/cecum	N/A	yes/yes/yes	5 / 7 / 9 [§]
134 [‡]	unknown (adult)	Female	ileum/sigmoid/cecum	N/A	yes/yes/yes	5 / 7 / 9 [§]

* - Frozen biopsies were also shipped to Baylor College of Medicine, where successful organoid lines were established using technique 1 and/or technique 3.
 † - Colon adenoma
 ‡ - Frozen biopsies were received from Turkey on dry ice.
 § - Days frozen includes two days on dry ice during international shipping.

C

Organoid lines generated using Technique 3

Patient ID	Patient Age (years)	Patient Sex	GI Region	Successful Enteroids (Fresh)	Successful Enteroids (Frozen)	Days Frozen at -80°C
148	49	Female	colon	N/A	yes	4
151	66	Female	colon	N/A	yes	6 or 13
150	64	Male	colon	N/A	yes	34 (-80/LN2)

D

Organoid lines generated using Technique 4

Patient ID	Patient Age (years)	Patient Sex	GI Region	Successful Enteroids (Fresh)	Successful Enteroids (Frozen)	Months Frozen in Liquid N ₂
81	49	Female	colon/duodenum	yes/yes	yes/yes	10
83	45	Female	colon	yes	yes	6
84	56	Female	colon	yes	yes	5
85	62	Female	colon	yes	yes	2.5
87	21	Male	colon/duodenum	yes/yes	yes/yes	7
88	33	Female	colon/duodenum	yes/yes	yes/yes	6
89	55	Male	colon/duodenum	yes/yes	yes/yes	12

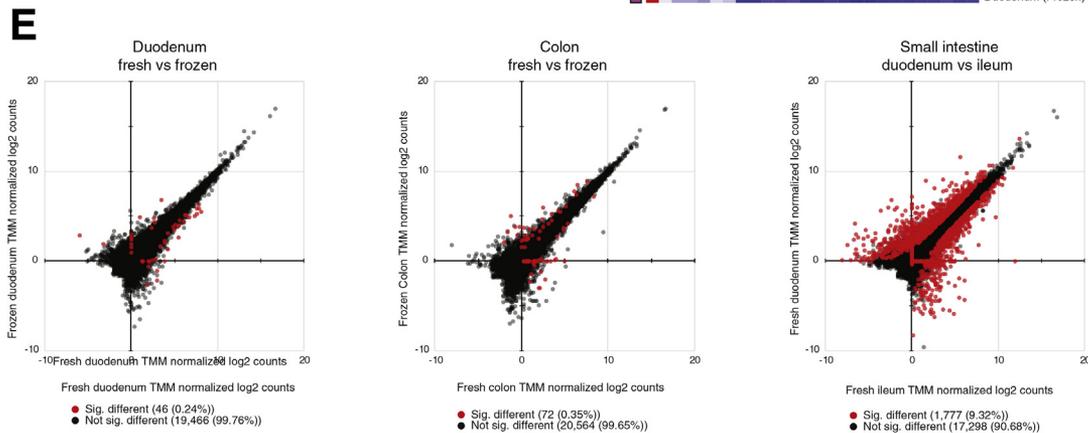
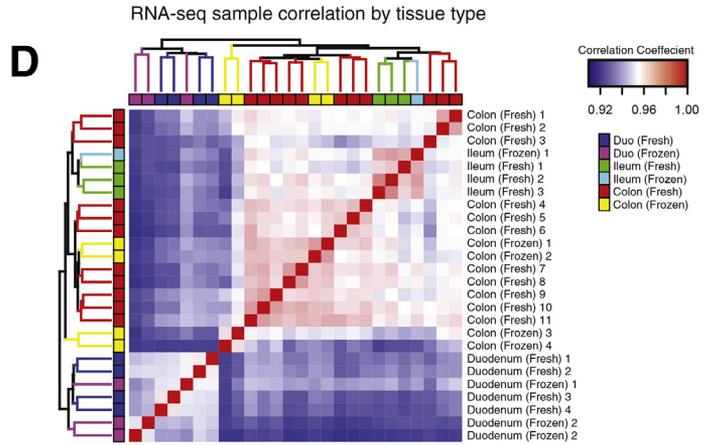
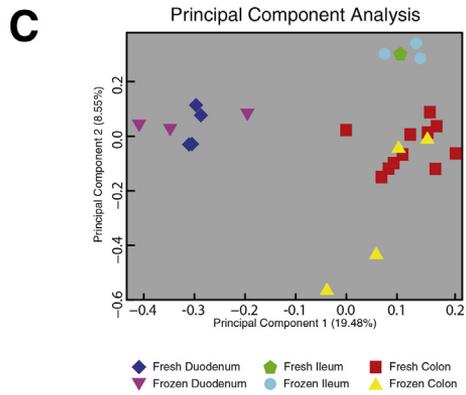
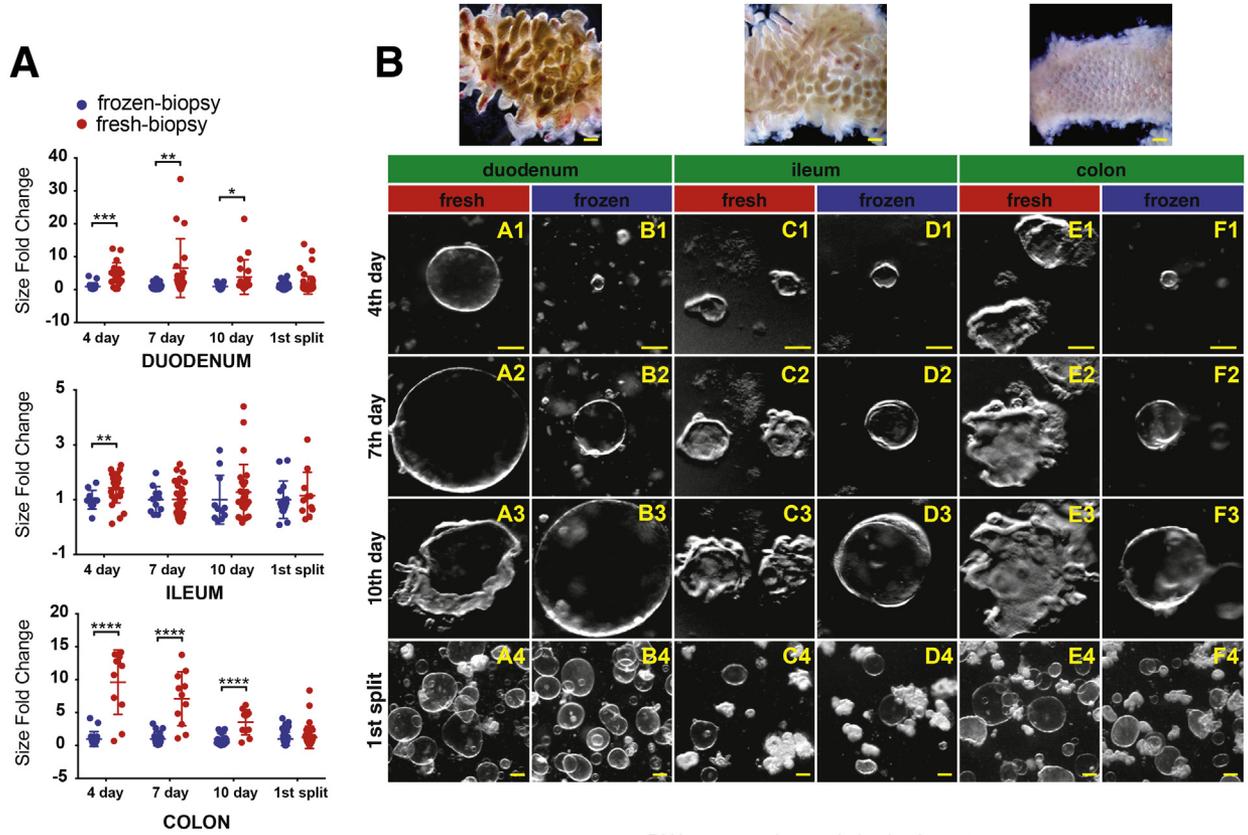
Figure 1. (continued)

and 10% dimethyl sulfoxide). By using a Mr. Frosty (ThermoFisher Scientific, Waltham, MA) cell-freezing container, the biopsy fragments were frozen to -80°C overnight. Both freezing media formulations performed equivalently (data not shown), thus we focused on the simple formulation. Upon thawing the cryopreserved biopsy specimens, 4 methods were developed for thawing and subsequent establishment of organoid cultures (Figure 1). Frozen vials were thawed at 37°C, the tissue was washed in recovery media (see

Supplementary Materials and Methods section, Figure 1). For techniques 1 and 3, tissue was partially digested with dispase or EDTA followed by mechanical perturbation to loosen adhesion between cell layers (Figure 1, Supplementary Figures 1–3).³ The epithelium was pelleted by centrifugation and then resuspended in recovery medium. For techniques 1 and 3, isolated epithelium was embedded in Matrigel (Corning, Bedford, MA) where it was cultured for 3 days in recovery media.⁸

We also tested a second thawing technique that omitted enzymatic digestion (technique 2) (Figure 1, Supplementary Figure 2). In this case we thawed the biopsy specimen, washed with recovery media, and embedded whole tissue fragments in Matrigel. These cultures were grown in recovery media for 1 week, where we observed epithelial cysts growing from the biopsy fragment (Supplementary Figure 2A). Tissue was removed from Matrigel and the epithelium was dissociated enzymatically using dispase,

Figure 2. (See next page). Organoids derived from fresh and frozen tissue appear morphologically indistinguishable after passage, and transcriptional profiles of organoids derived from fresh and frozen biopsy specimens are nearly identical. (A) The size of organoids derived from different sections of intestine were measured at 4, 7, and 10 days of growth and after the first split. * $P < .05$, ** $P < .01$, *** $P < .005$, **** $P < .001$. (B) Organoids derived from duodenum (representative biopsy specimen shown at top left, organoids columns A and B), ileum (biopsy top middle, organoids columns C and D), and colon (representative biopsy specimen shown at top right, organoids columns E and F) have different morphologic characteristics when derived from fresh tissue (A1–A4, C1–C4, and E1–E4). Duodenum-derived organoids grow as smooth cystic structures regardless of whether they originate from fresh or frozen tissue (A1–A4 and B1–B4). Organoids from fresh ileum and colon grow with distinct budded morphology whereas those derived from patient-matched frozen tissue (D1–D4 and F1–F4) lack these morphologic distinctions during the early phases of culture establishment. After the first passage and for the remainder of the observed lifetime of the culture, fresh and frozen organoid morphologies cannot be distinguished (A4–F4). (C and D) Clustering organoid RNA sequencing samples by (C) source of variance with principal component analysis or by (D) sample similarity using Pearson correlation. Both analyses generate groups from the same gastrointestinal region regardless of derivation from fresh or frozen tissue biopsy specimens. (E) Differential expression analysis comparing fresh-derived duodenum organoids with frozen-derived duodenum organoids (left), or fresh-derived colon organoids with frozen-derived colon organoids (middle), show a near lack of significant differences in gene expression. In marked contrast, comparing organoids from different regions of the small intestine, such as duodenum and ileum (right), yields more than 9% of genes showing significantly different expression. *Significant difference denotes an adjusted P value of $\leq .01$ and fold difference ≥ 2 (log2 fold difference ≥ 1 or ≤ -1). Biopsy images are representative and not necessarily tissue-matched to the subsequent organoid images.



similar to technique 1, and cultured further (Figure 1). We also found that whole fresh crypts could be isolated, cryopreserved, and recovered to generate organoids (technique 4) (Figure 1, Supplementary Figure 2C).

By using techniques 1 and 2, we established 20 frozen biopsy-derived organoid lines from 4 different regions of the GI tract (stomach, duodenum, ileum, and colon) from healthy patients, and 1 organoid line from adenomatous tissue (Figure 1B). We generated 3 patient-specific organoid lines using technique 3 (Figure 1C), and 7 patient-specific organoid lines using technique 4 (Figure 1D). All techniques had a 100% success rate, showing the robustness of the method. The initial growth of organoids from frozen samples is delayed when compared with freshly isolated epithelium (Figure 2A), but frozen organoids eventually catch up with fresh tissue organoids and are indistinguishable after the first passage (Figure 2A and B).

To determine if cryopreservation caused any molecular differences within the organoids, we performed RNA sequencing on a cohort of fresh tissue organoids ($n = 10$) and cryopreserved tissue-derived organoids ($n = 5$). Principal component analysis showed that the strongest driver of variability was the GI region from which organoids were derived (Figure 2C). Similarly, using Pearson correlation, fresh and frozen organoids from the same region of the intestine clustered together, and there was no clustering based on fresh vs frozen status (Figure 2D). Finally, we performed differential expression analysis to compare organoids derived from fresh vs frozen biopsy specimens from the same region of the intestine (ie, duodenum or colon).⁹ Strikingly, frozen and fresh organoids were nearly identical, with only 46 (0.24% of all genes expressed) and 72 genes (0.35% of all genes expressed) showing significant expression differences in the duodenum and colon, respectively (\log_2 fold change ≥ 1 or \log_2 fold change ≤ -1 ; $P \leq .01$) (Figure 2E). By contrast, comparing duodenum vs ileum using the same method showed 1777 (9.32% of all

genes expressed) differentially expressed genes (Figure 2E).

Organoid generation from cryopreserved biopsy specimens was robust and patients ranged in age from 2 to 70 years and included both males and females. We kept a subset of samples frozen for times ranging from 3 days to 10 months with successful organoid cultures derived in each case (Figure 1B–D). To test the idea that cryopreserved biopsy specimens can be shipped long distances, 2 patient samples were cryopreserved at the University of Michigan and shipped on dry ice to Baylor College of Medicine, where organoid cultures were established successfully from both patients (Figure 1, samples 104 and 105). Biopsy specimens from 3 patients were shipped on dry ice from Ankara, Turkey, to the University of Michigan, where organoids were established successfully from these samples (Figure 1B, samples 132, 133, and 134). These results indicate that GI tissue can be cryopreserved, shipped long distances, and cultured from a diverse human demographic.

In summary, we have shown human biopsy specimens from multiple regions of the GI tract can be cryopreserved and, upon thawing, used to establish long-term organoid cultures. We speculate that this technically simple process will be adaptable to biopsy specimens from other organs/tissues, and that the major obstacle will be the identification of tissue-specific recovery conditions after thawing. Frozen tissue samples now can be shipped across the globe, effectively freeing patients, hospitals, clinics, researchers, and diagnostics laboratories from the necessity of geographic proximity.

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***Authors share co-first authorship.**

Abbreviation used in this letter: GI, gastrointestinal

 **Most current article**

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Author contributions

Michael Czerwinski, Yu-Hwai Tsai, John Y. Kao, and Jason R. Spence conceived the study; Yu-

Hwai Tsai, John Y. Kao, Michael Czerwinski, Angeline Wu, Lauren Marie Nowacki, Peter D. R. Higgins, Michael K. Dame, Durga Attili, Evan Hill, and Justin A. Colacino conducted the experiments; John Y. Kao and Peter D. R. Higgins obtained study permissions and collected patient samples; Yu-Hwai Tsai, Michael Czerwinski, Angeline Wu, Jason R. Spence, Lauren Marie Nowacki, Noah F. Shroyer, John Y. Kao, Peter D. R. Higgins, Michael K. Dame, Durga Attili, Evan Hill, and Justin A. Colacino analyzed the data; Michael Czerwinski and Jason R. Spence wrote the manuscript; and all authors edited the manuscript and approved the final version.

Conflicts of interest

The authors disclose no conflicts.

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Supplementary Materials and Methods

Laboratory style step-by-step protocols with reagent ordering information is provided.

Media Formulations

LWRN growth media is produced as previously described (see the [Supplementary Appendix](#) for protocols).⁴ Conditioned media from L-WRN cells containing Wnt3a, Rspodin3, and Noggin was mixed 1:1 with 2× basal media comprising 214 mL advanced Dulbecco's modified Eagle medium/F12, 5 mL GlutaMAX (Gibco, Japan) (100×, 200 mmol/L), 5 mL HEPES (100×, 1 mol/L), 5 mL N2 supplement (100×), 10 mL B27 supplement (50×), 5 mL penicillin/streptomycin (100×), 1 mL N-acetylcystine (500 mmol/L), and 5 mL nicotinamide (1 mol/L).

Recovery media consisted of LWRN growth media supplemented with Thiazovivin (2.5 μmol/L), SB431542 (100 nmol/L), CHIR99021 (4 μmol/L), and Y27632 (10 μmol/L). This media was used for initial thawing as well as the first 24 hours of culture to inhibit apoptosis in the thawed cells. After the first 24 hours, recovery media minus Y27632 was used for the next 48 hours.

Organoid Culture

Once established from frozen or fresh tissue, all organoids were cultured as previously described.⁴ Media changes occurred every 2 days with LWRN growth media.

Biopsy Collection and Cryopreservation

All human sample collection was performed in accordance with the University of Michigan Institutional Review Board regulations and were collected under protocols HUM00041845 and HUM00039121. Samples received from Turkey were approved under protocol HUM00137277, and studies conducted at Baylor College of Medicine were performed under protocol H-35094. Human tissue biopsy specimens were collected via endoscopy from the stomach, colon, distal ileum, and

duodenum. Biopsy specimens were stored on ice in sterile saline after initial collection and before transport to the laboratory. To remove potential contaminants, all biopsy specimens were washed 3 times with sterile phosphate-buffered saline or with phosphate-buffered saline plus antibiotics (penicillin/streptomycin or gentamicin). Large biopsy specimens then were cut into pieces no larger than 3 mm² and resuspended in 1 mL freezing media (Dulbecco's modified Eagle medium/F12, 10% fetal bovine serum, 10% dimethyl sulfoxide) each in individual cryovials. Slow freezing was accomplished with freezing containers (5100-0001; ThermoFisher, Waltham, MA) filled with 250 mL isopropanol placed at -80°C overnight. For long-term storage, frozen biopsy specimens then were moved to liquid nitrogen storage.

Establishment of Organoid Cultures From Frozen Biopsy Specimens

Cryovials containing biopsy specimens were thawed rapidly by submerging the lower half into a 37°C water bath. Freezing media then gently was removed from thawed biopsy specimens and replaced with recovery media. Biopsy specimens then were transferred to a Petri dish and minced into smaller pieces with a sterile scalpel. To separate the epithelium from the underlying cell layers, minced biopsy specimens then were incubated in dispase (07923; STEMCELL Technologies, Vancouver, Canada) for 30 minutes on ice. Dispase then was removed and replaced with 100% fetal bovine serum for 15 minutes on ice. To mechanically separate the tissue layers, a volume of advanced Dulbecco's modified Eagle medium/F12 (12634010; Gibco, Grand Island, NY) equal to the initial volume of fetal bovine serum was added to the biopsy tissue before vigorously pipetting the mixture several times. Epithelial fragments then settled to the bottom where they were collected manually on a stereoscope by pipet. The epithelium then was washed with ice-cold advanced Dulbecco's modified Eagle medium/F12 and allowed to settle to the bottom of a 1.5-mL tube. The media then was

withdrawn from the loose tissue pellet and replaced with Matrigel. The Matrigel containing the isolated epithelium then gently was mixed to suspend the cells evenly before being pipetted into individual 50-μL droplets in a 24-well plate. The plate containing the droplets then was incubated at 37°C for 15 minutes to allow the Matrigel to solidify before adding LWRN growth media containing Thiazovivin (2.5 μmol/L), SB431542 (100 nmol/L), CHIR99021 (4 μmol/L), and Y27632 (10 μmol/L). After 24 hours, the media was replaced with LWRN growth media containing TZV (2.5 μmol/L), SB431542 (100 nmol/L), and CHIR99021 (4 μmol/L). After 3 days, cultures then were maintained with LWRN growth media replaced every other day.

RNA Sequencing Analysis

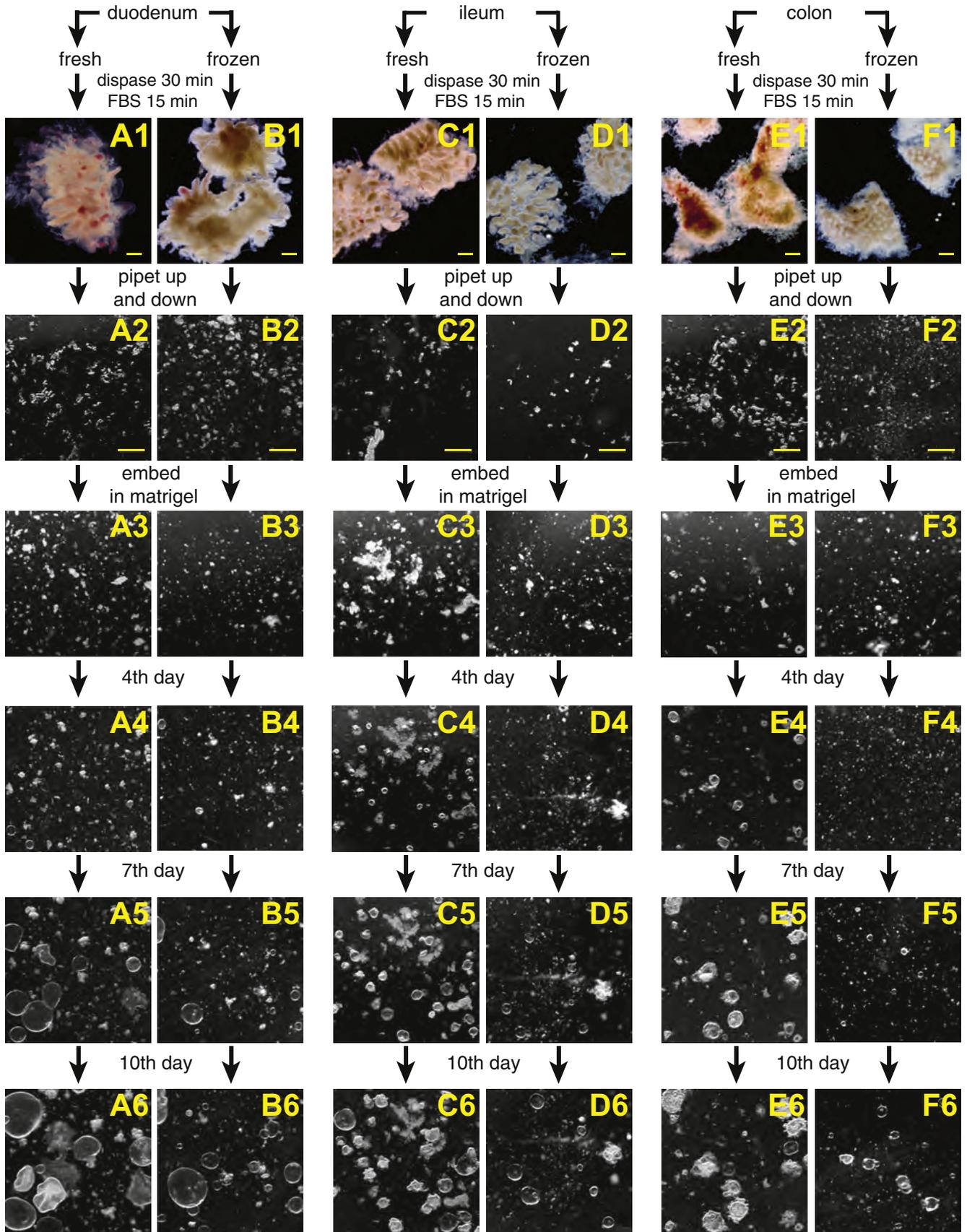
Early passage organoids were collected and sorted manually to remove those that were dead or unhealthy, with multiple organoids pooled together within each replicate. Organoid samples and replicates used for RNA sequencing analysis were derived from patients: 98 (2 fresh colon, 2 frozen colon), 99 (1 fresh colon), 101 (2 fresh colon), 102 (2 frozen colon), 103 (2 fresh colon), 104 (2 fresh colon, 2 fresh ileum), 105 (2 fresh colon, 1 fresh ileum, 1 frozen ileum), 115 (2 fresh duodenum, 1 frozen duodenum), and 117 (2 fresh duodenum, 2 frozen duodenum) ([Figure 1B](#) shows patient data). RNA from each sample was isolated using MagMAX-96 Total RNA (AM1830; Applied Biosystems), RNA isolation kits, and used as input for library generation with Takara SMARTer Stranded Total RNA Sample Prep Kit (634876; Takara Bio USA). A total of 26 samples were sequenced for 50-bp single-end reads across 10 lanes on an Illumina HiSeq 2500 by the University of Michigan DNA Sequencing Core. All reads were quantified using Kallisto pseudo alignment to an index of transcripts from all human genes within the Ensembl GRCh38 database.¹² Gene level data then were generated from the Kallisto estimated counts per transcript using the Bioconductor package tximport.¹³ Differential

expression analysis was performed using the Bioconductor package DESeq2 and the gene level count data. A gene was considered to be differentially expressed if it had a 2-fold or larger difference between groups and an adjusted *P* value of .01 or less.

Principal component analysis and sample clustering were performed in R with log2 transformed and centered gene counts of gene level data on all genes that had a sum of at least 10 counts across all samples. Replicates for all samples were clustered by

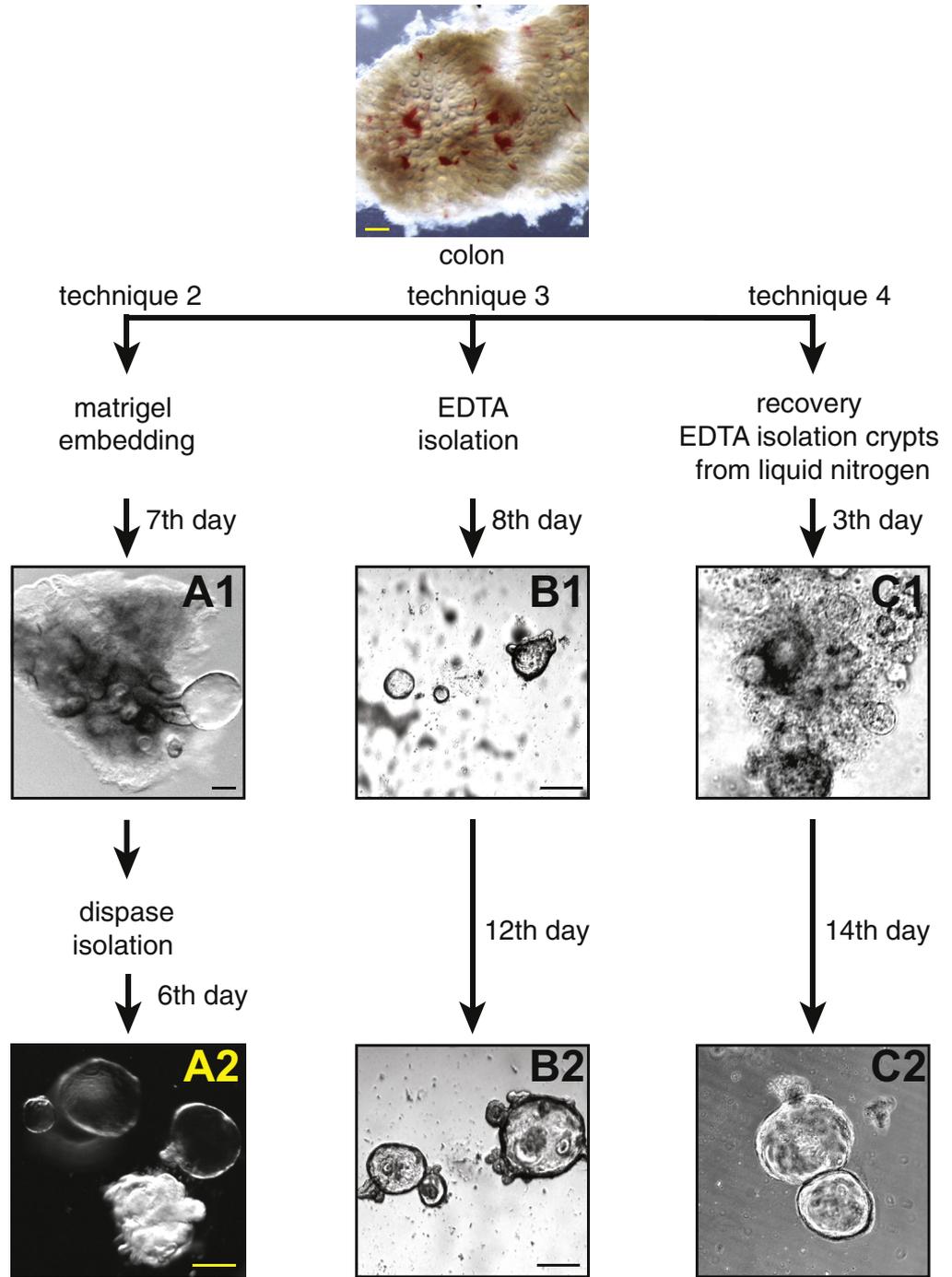
Euclidian distance, and pairwise Pearson correlation coefficients were plotted in R. All reads are deposited at the EMBL-EBI ArrayExpress archive under accession E-MTAB-6516.

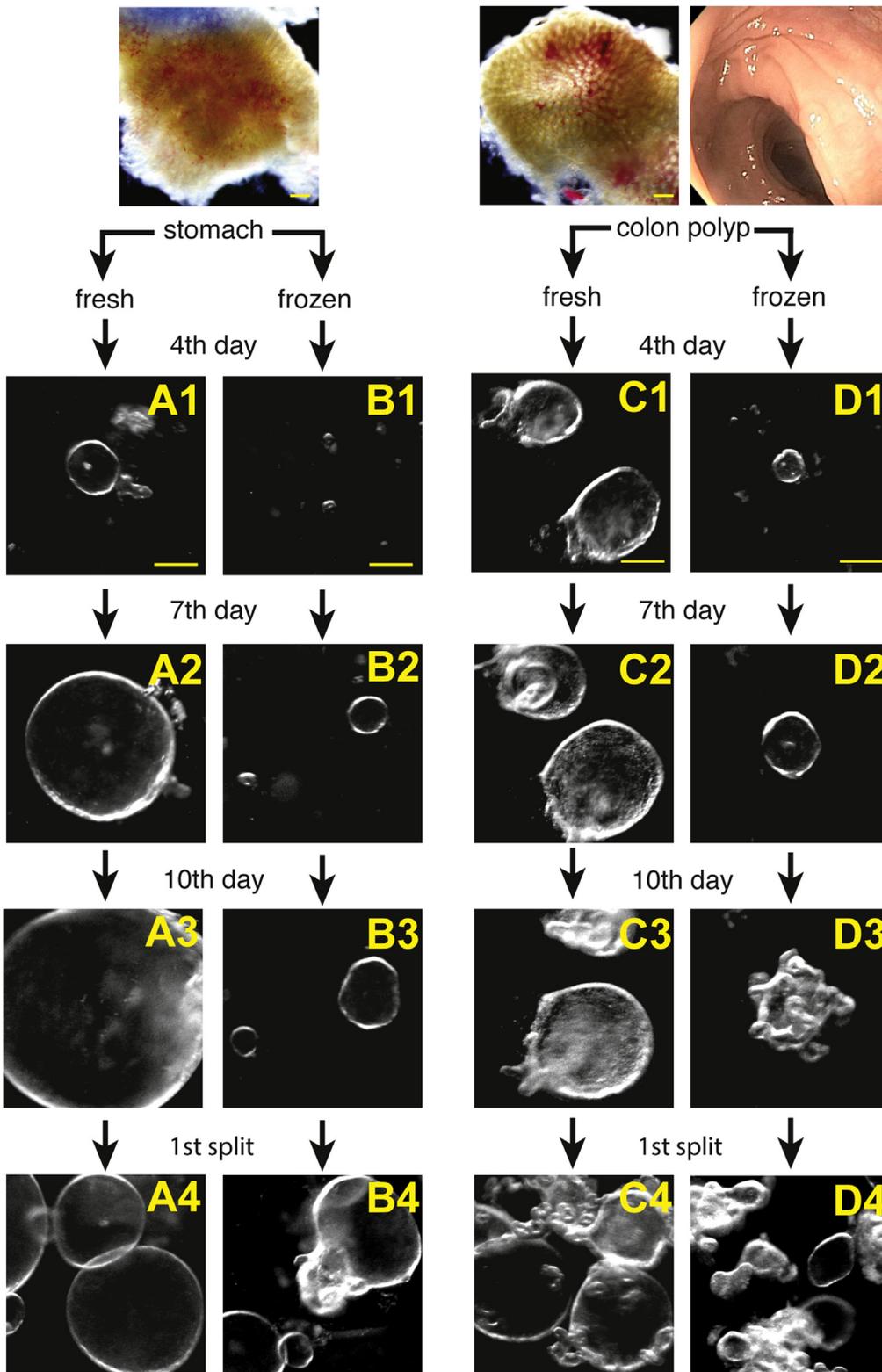
For detailed step-by-step protocols see the [Supplementary Appendix](#).



Supplementary Figure 1. (See *previous page*). **Organoids derived from frozen tissue have delayed initial growth characteristics.** Biopsy specimens from duodenum (columns A and B), ileum (columns C and D), and colon (columns E and F) can be used to derive intestinal organoids before and after freezing. Epithelial fragments from fresh biopsy specimens (A2, C2, E2) are larger and contain recognizable crypts compared with the small fragments from frozen (B2, D2, F2) samples. Growth kinetics are delayed in cultures established from frozen tissue (B2–B5, D2–D5, F2–F5) relative to fresh tissue (A2–A5, C2–C5, E2–E5), but attain normal growth kinetics and size by 10 days in culture (A6–F6). Biopsy images are representative and not necessarily tissue-matched to the subsequent organoid images.

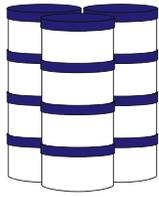
Supplementary Figure 2. Four methods are developed that allow successful generation of patient biopsy specimens. Technique 2 (left; A1 and A2) adds a step relative to technique 1 in which the entire biopsy specimen is embedded in Matrigel and allowed to repair from the freezing process before tissue digestion and crypt isolation. Technique 3 (middle; B1 and B2) is very similar to technique 1 but uses a gentle EDTA treatment to separate the epithelium from the mesenchyme. In technique 4 (right; C1 and C2), whole crypts were isolated, subsequently cryopreserved, and recovered to generate organoids. All 4 techniques result in pure organoid cultures approximately 2 weeks after initially thawing the biopsy specimen. Biopsy images are representative and not necessarily tissue-matched to the subsequent organoid images.





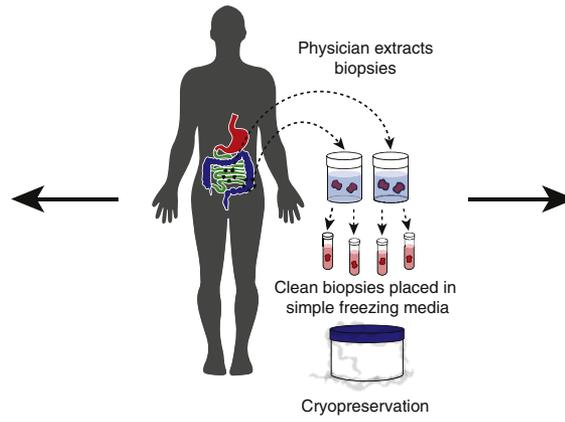
Supplementary Figure 3. Organoids can be derived from frozen biopsy specimens of stomach and colon adenoma. Biopsy specimens from stomach (columns A and B) and colon adenoma (columns C and D) can be used to derive organoids before and after freezing. Organoids from fresh biopsy specimens (A1–A3, C1–C3) are larger compared with the small organoids from frozen (B1–B3, D1–D3) samples, but attain normal growth kinetics and size after the first split for both organoids (A4–B4, C4–D4). Biopsy images are representative and not necessarily tissue-matched to the subsequent organoid images.

Tissue Biobank

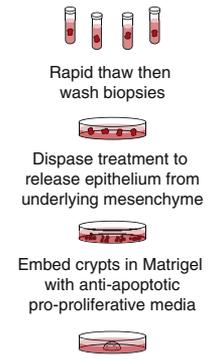


Long term storage for:
Research material
Regenerative Medicine
etc...

Clinical procedures



Laboratory procedures



Supplemental Graphical Summary.