

Guiding Principles: Current Practices and Considerations for Benchmarking Human Gastrointestinal Organoids

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SUMMARY

This commentary discusses best practices and considerations for benchmarking gastrointestinal organoids against native GI tissues. It focuses on biological scales and concepts relevant to targeted and unbiased organoid benchmarking, reviewing examples from single-cell RNA-seq studies as a framework for addressing emerging questions in the field.

Since the first reports of organoids cultured from primary human small intestine and colonic epithelium in 2011, these systems have held considerable potential to accelerate our understanding of gastrointestinal biology and disease. Improvements in tissue isolation and culture conditions for primary tissue organoids, as well as the parallel development of protocols for generating organoids from pluripotent cells, have made organoid research increasingly accessible over the last 15 years. As organoids emerge as bona fide model systems, there is a growing need to understand and evaluate the accuracy and limitations of their ability to represent different aspects of gastrointestinal biology, both within and across laboratories and experimental contexts. In this *Guiding Principles* commentary, we outline the current state of the field and challenges for benchmarking human small intestinal and colonic organoids against native gastrointestinal tract biology. We outline a conceptual framework for approaching organoid benchmarking at multiple scales, review current approaches and efforts in assaying similarities between organoids and their source tissues, and discuss the “next

frontiers” for validating organoids as reproducible and high-fidelity models of the human gastrointestinal tract. (*Cell Mol Gastroenterol Hepatol* 2026;■:101832; <https://doi.org/10.1016/j.jcmgh.2026.101832>)

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Over the past decade, organoid models of the human intestine and colon have gained increasingly broad applications, transitioning their role in gastrointestinal (GI) research from that of a niche method in stem cell biology to that of a bona fide model system. Recent studies have leveraged the complex cellular diversity of human GI organoids to identify mechanisms of human intestinal development and regeneration,^{1,2} engineer platforms for drug screening,^{3,4} or identify evolutionary changes in gut

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Abbreviations used in this paper: ASC, adult stem cell; ATAC-seq, assay for transposase-accessible chromatin using sequencing; CUT&RUN, Cleavage Under Targets and Release Using Nuclease; CUT&TAG, Cleavage Under Targets and Tagmentation; D, dimensional; DISSECT, Disaggregation for Intracellular Signaling in Single Epithelial Cells from Tissue; EEC, enteroendocrine cell; GI, gastrointestinal; MC, mass cytometry; PSC, pluripotent stem cell; scRNA-seq, single-cell RNA sequencing.

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biology.⁵ As their use expands, it becomes important to consider a challenge common to all model systems: how accurately do GI organoids recapitulate human biological processes and disease? Understanding how closely organoids resemble in vivo GI biology will allow the research community to confidently apply these models to functional and mechanistic questions that would be otherwise impossible to address in humans.

This *Guiding Principles* commentary is the second in a series of 3, inspired by user input from the GI organoid research community, and intended to open community-wide discussions regarding emerging challenges in the field.⁶ Validating biological characteristics of human GI organoid models against in vivo tissue (referred to throughout as “benchmarking”) was consistently recognized as an important consideration. At the same time, participants expressed concerns about the potential of “strict” benchmarking expectations to restrict entry to or innovation in the field, as well as practical concerns including time and cost barriers to benchmarking. Rather than prescribing a “one size fits all” approach to benchmarking, this commentary is intended to highlight: (1) community recognition of the importance of benchmarking; and (2) growing interest in approaches that support rigor while accommodating the diversity of experimental contexts and stages of innovation within GI organoid research. We review the current state of GI organoid benchmarking against native tissue, which to date has relied heavily on matching gene expression states at the cellular level since single-cell RNA sequencing (scRNA-seq) has become a widely used characterization method. We also highlight measurements at different biological scales that provide avenues to benchmark different aspects of GI function outside of gene expression (Figure 1). Although many benchmarking concepts are broadly applicable across in vitro human tissue models and have been reviewed elsewhere,⁷ this commentary focuses on adult stem cell (ASC) or “primary” and pluripotent stem cell (PSC)-derived small intestinal and colonic organoids. We define this broadly to include 3-dimensional (D), 2D, and bioengineered systems.

What Is Benchmarking?

For many researchers using model systems, benchmarking is a familiar practice. In practical terms, benchmarking involves confirming that assumptions about organoid biology are relevant to the in vivo processes being modeled. Early examples of GI organoid benchmarking include the first studies on ASC-derived small intestinal and colonic organoids, where the presence of all cellular lineages found in vivo were validated using standard markers by immunohistochemistry.⁸ More recent studies validated regional identity of enteroendocrine cells (EECs) in ASC-derived small intestinal organoids by reverse transcription-quantitative polymerase chain reaction to measure hormone expression in known proximal-to-distal patterns.⁹ We define this scale of benchmarking as “targeted benchmarking,” which commonly assesses whether organoids exhibit expected and defining features specifically relevant to the central biological aims of a study. Minimal targeted benchmarking should at least confirm that organoids match

their tissue of origin. This can include basic validation of regional identity (eg, that colon-derived organoids correspond to colon tissue and ileal organoids to ileum) using defined markers, because ASC-derived organoids typically retain regional identity of their origins.^{10–13} Consistent application of targeted benchmarks is essential to ensure rigor and reproducibility across studies. Because targeted benchmarking is inherently context-dependent and shaped by both specific experimental requirements and existing or emerging knowledge of native GI tissues, it is not easily generalized and should be tailored to the scientific goals of individual studies.

The expansion of GI organoid applications has paralleled the rise of single cell -omics assays, particularly scRNA-seq. These advances have facilitated technically complex and resource-intensive studies evaluating organoids from different tissue sources, pathophysiological contexts, or culture formats in an unbiased manner. In contrast to targeted benchmarking, these studies often apply “unbiased benchmarking” to assay or improve the overall fidelity of organoid systems for modeling in vivo biology. One of the critical strengths of organoids is their ability to recapitulate the cell type heterogeneity seen in vivo. Thus, unlike bulk characterization, single-cell resolution and unbiased readouts are important to: (1) avoid confounders associated with averaging signals from multiple cells; and (2) account for biological features not defined a priori (Figure 1). Unbiased benchmarking can establish broadly relevant principles and generate openly accessible datasets, but its considerable time and resource costs limit routine inclusion in many GI organoid studies. Because unbiased benchmarking represents an evolving conceptual and technical space that will require community-wide buy-in to maximize data utility, it is the primary focus of this commentary.

Unbiased benchmarking of GI organoid systems against native tissues requires structured approaches to understand not only similarities, but also ways in which organoids differ. Using frameworks from systems and computational biology, unbiased benchmarking integrates molecular, cellular, and functional measurements to map these differences across biological scales (Figure 1). Rather than simply treating “mismatches” as shortcomings, unbiased benchmarking can also highlight specific deviations between in vivo references and organoids, such as altered cell states, gene expression programs, and cell-cell interactions that shape organoid system behavior. Identifying these differences can define the appropriate scope of biologically grounded questions and strengthen interpretations of results from organoid experiments, enabling meaningful use of these systems in studying GI biology. Benchmarking studies that generate -omic scale data also enable us to identify necessary components for tailoring new organoids systems to different biological questions (Box 1).

Current State of Gastrointestinal Organoid Benchmarking

scRNA-seq provides an unbiased and high-resolution view of gene expression across individual cells. It is well-

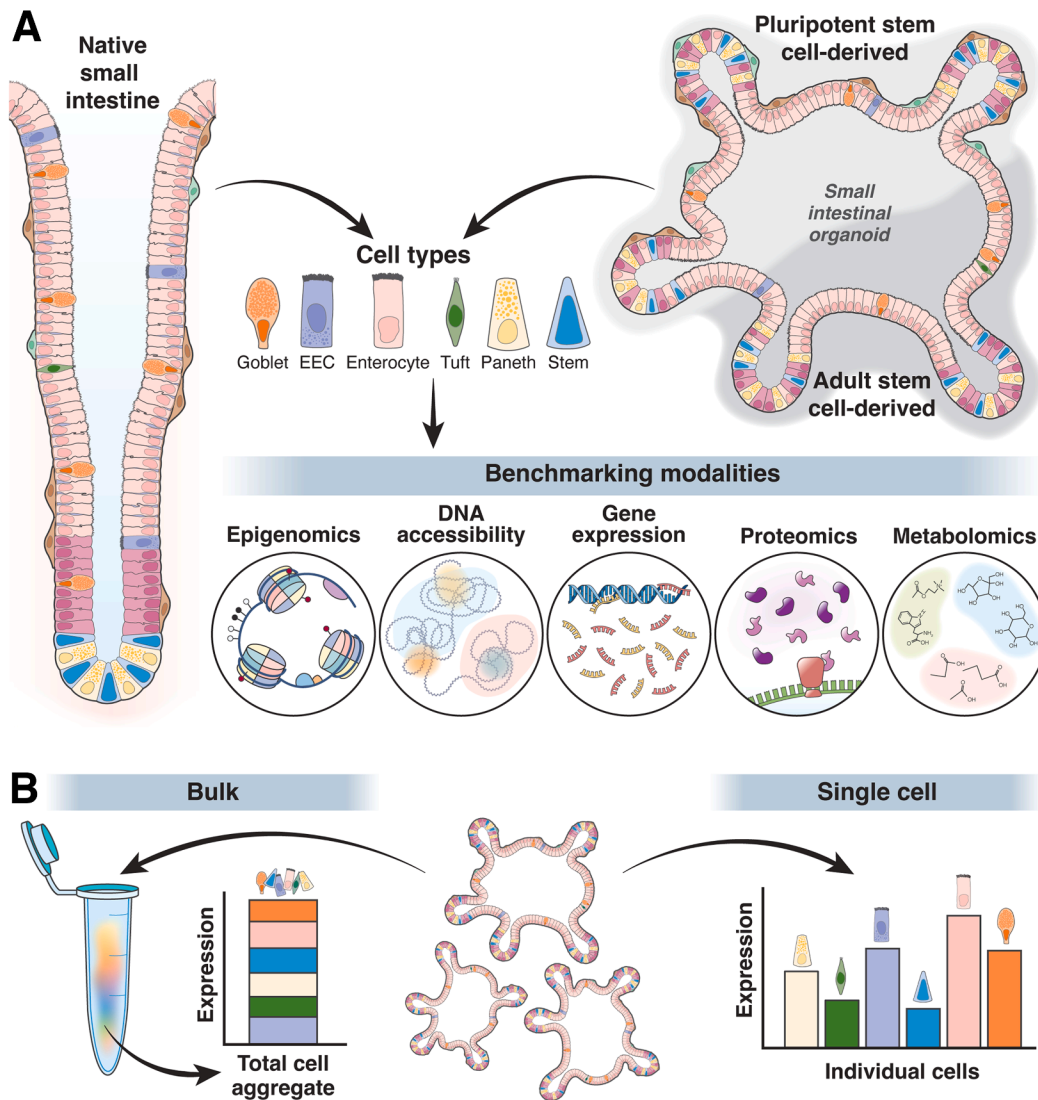


Figure 1. Benchmarking human GI organoids across scales. Comparing native GI tissue with organoid models requires multiple approaches that capture different aspects of small intestinal and colonic function. The crypt–villus axis of the small intestine, with a lack of villi in the colon not represented here, as compared with organoids, shows differences in architecture or cell types that can be ascertained using classical imaging techniques. Beyond morphology, assessments of DNA accessibility, epigenomics, gene expression, proteomics, and metabolomics probe distinct aspects of GI and organoid functionality (A). Many of these biological scales have methods that can be performed at either bulk or single-cell resolution: bulk analyses capture overall tissue or organoids characteristics, whereas single-cell approaches reveal cell-specific changes that can be masked by bulk measurements (B).

Box 1. Guiding Principle: Tailor Consistent Benchmarking Approaches to Study-Specific Goals

GI organoid benchmarking is best approached without reliance on universal frameworks that may restrict innovation or impose resource-dependent barriers limiting inclusion of organoids in study design. Targeted assays, such as immunohistochemistry or reverse transcription-quantitative polymerase chain reaction, are appropriate and often sufficient to validate study-specific organoid benchmarks that ensure biological relevance. Omics approaches facilitate unbiased benchmarking, which can generate broadly applicable biological insights and foundation data sets for GI organoid biology.

suited for systematic comparisons between in vivo reference tissue and organoid models, and understanding its application can provide a useful framework for approaching unbiased benchmarking of GI organoids. scRNA-seq has been used in several studies incorporating organoids derived from ASCs,^{14–16} fetal tissues,¹⁷ or PSCs,¹⁸ and comparing these with their corresponding in vivo tissues. As many atlas-scale studies of human tissue focus on scRNA-seq data as a shared reference space,^{19–21} scRNA-seq has emerged as a central tool for evaluating similarities and differences between organoids and native tissues. Although scRNA-seq cannot benchmark all biological scales relevant to the GI tract (Figure 1), understanding its conceptual and technical application can provide researchers with a framework for approaching other aspects of GI organoid benchmarking.

Benchmarking Gastrointestinal Organoids Through Gene Expression

Assaying gene expression is commonly used for benchmarking GI organoids against native tissue. Given the limitation of bulk RNA sequencing to directly identify different cell types present in native tissue, scRNA-seq represents the most widely adopted unbiased approach. Approaches for scRNA-seq analysis range from simply assessing cluster overlap between organoid and tissue cell populations,¹⁴ to complex quantitative analyses involving nested approaches that evaluate cell-type projection probability followed by overall tissue alignment.¹⁵ Many studies focus on first assigning cell identities, often using automated annotation tools such as CellTypist alongside manual marker-based approaches.²² This is followed by evaluating cell-type proportions between in vivo references and organoids and comparing transcriptional profiles

(Figure 2A).^{17,23–28} Notably, differences in cell-type composition between organoids and tissue are frequently observed, with organoids often enriched for stem-like or progenitor populations, likely reflecting standard culture conditions that favor these cell states.²⁹ Major transcriptomic differences can also be observed, for example, Yang et al found that organoid enterocyte populations cluster separately from in vivo enterocytes, indicating baseline discrepancies between organoids and tissue.¹⁶ These efforts can also identify minimal gene panels for more cost-effective targeted benchmarking to assess organoid-specific deviations from in vivo biology.

Beyond cell type assignment, benchmarking can incorporate differential gene expression analyses to identify cell types, signaling pathways, and regulatory programs that are systematically unchanged or up- or down-regulated in organoids relative to tissue (Figure 2B).^{25,26} Differential gene expression in small intestinal organoids, as

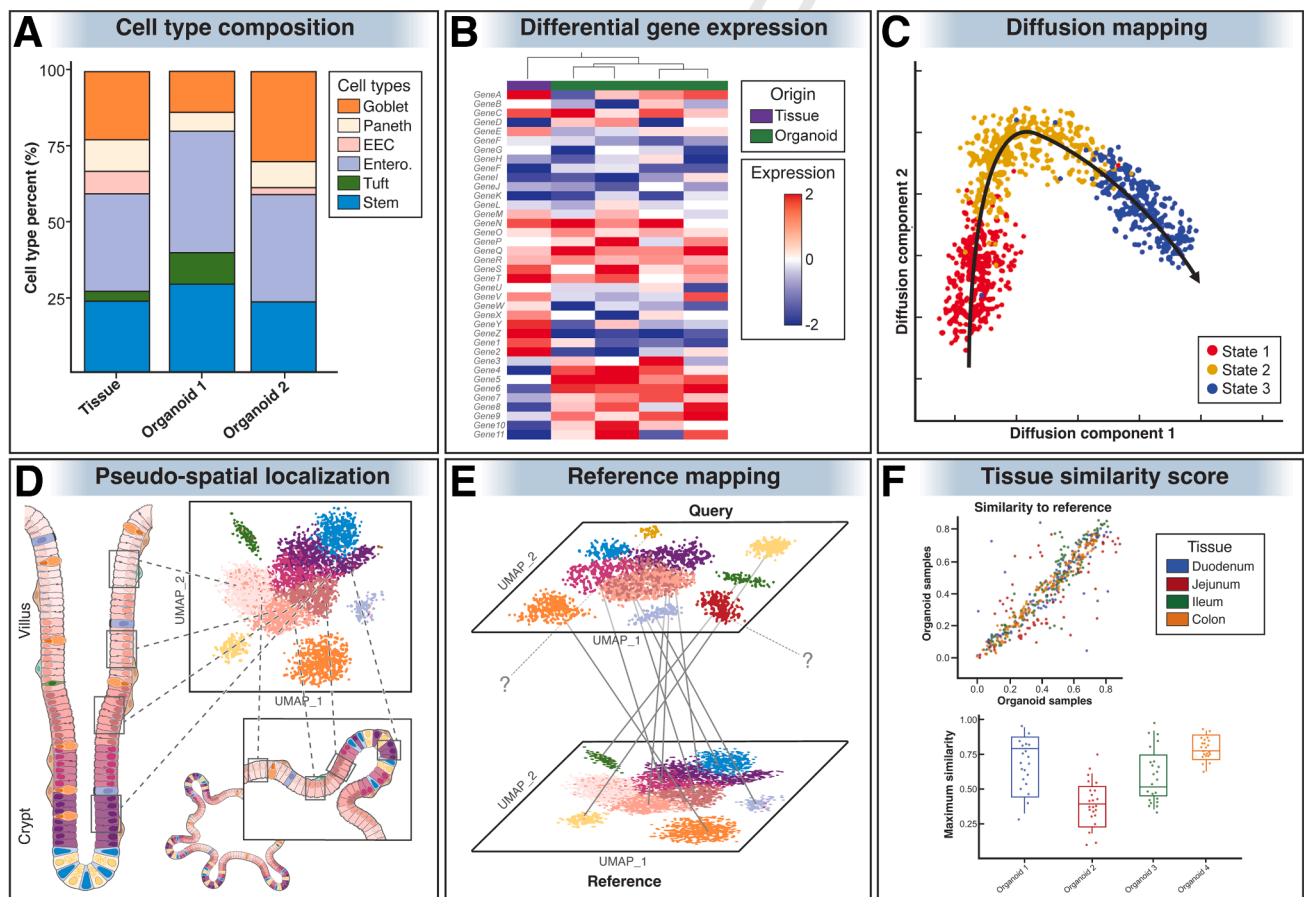


Figure 2. Strategies to implement scRNA-seq to benchmark GI organoids. Implementing scRNA-seq to organoid benchmarking can be achieved via various tools and combinatory approaches. (A) Defining cell identities and population enrichment between in vivo and ex vivo or across models. (B) Differential gene expression analysis to identify variations in cell state and identity through regulatory pathways and transcriptional programs. (C) Inferring organoid differentiation over diffusion mapping or pseudotime analysis in comparison to in vivo differentiation. (D) Pseudo-spatial localization by predicting cell position over the crypt–villus axis based on their transcriptomic profile. (E) Quantitative mapping of a query data over reference Principal Component Analysis or fixed reference dataset. (F) Similarity score for cell type and transcriptomic profile to summarize dataset harmonization and similitude to in vivo. UMAP, Uniform Manifold Approximation and Projection.

demonstrated by Smith et al in 2025, revealed hormone and receptor expression patterns in ASC-derived organoid EECs, enabling the identification of specific EEC subtypes that closely match in vivo EEC transcriptomic profiles.¹⁴ Wang et al, in 2022, observed that colorectal organoids, whether derived from normal or cancer tissue, upregulated tumor-associated marker CEACAM6 when compared with matched in vivo reference, highlighting culture-induced shifts in cell states.²⁶ These comparisons can be further interpreted through Gene Set Enrichment Analysis,²⁵ linking observed transcriptional changes to functional pathways and biological processes, and differences in tissue function. Such marker-, gene-, and pathway-based comparisons can be extended across all cell types, enabling quantitative comparisons of cellular composition between organoids and native tissue. Because organoids are typically derived from stem cells, assessing their developmental trajectories can provide significant insight into their correspondence to native tissue development. One common approach is placing cells along inferred trajectories using diffusion mapping, as in Figure 2C, or pseudotime analysis to compare organoids against known in vivo differentiation tracks.^{27,28} This can include predicting each organoid cell's position along the in vivo crypt-villus axis based on known transcriptomic changes as cells migrate towards the lumen (Figure 2D), providing a spatially informed measure of differentiation.^{17,30} These analyses benchmark not only static cell identity, but also the dynamic progression of organoids through developmental pseudotime.

More complex strategies employ quantitative mapping that can be applied to tissue-matched organoid and reference data generated in parallel to mitigate biological variability.^{23,24,31,32} Quantitative mapping involves the application of computational algorithms to identify and “label” cell populations in one dataset based on predefined cell populations in a reference dataset. It provides a systematic framework for benchmarking organoids against native tissues across patients and public datasets. Alternatively, organoid scRNA-seq data can be mapped to existing public datasets, lowering costs and barriers to entry while facilitating benchmarking and cross-study reproducibility. For example, recent studies leveraged a public scRNA-seq fetal intestinal dataset to benchmark PSC-derived organoids using quantitative mapping, which found that transplanted PSC-derived organoids share similar developmental trajectories and transcriptomic signatures to fetal intestine.¹⁸ Practical implementations of quantitative mapping include Seurat's MapQuery,³³ which projects query cells into the reference Principal Component Analysis space and assigns cell identities via anchor-based weighted voting, and scArches,³⁴ which maps new datasets onto a fixed reference using transfer learning (Figure 2E). Alternative frameworks for label transfer and mapping include ELLeFhAnt,³⁵ which uses ensemble machine-learning classifiers to assign cell-type labels, and scANVI,³⁶ a semi-supervised generative model that harmonizes datasets and predicts probabilistic cell-type labels. Simple correlation scores can summarize overall transcriptional similarity of each cell type, whereas more sophisticated approaches

can define an integrative tissue similarity score (Figure 2F) (miloR³⁷ and scrabbtr³⁸). This approach enables a direct comparison between transcriptomic neighborhoods in organoids and those in tissue while also reducing error from variability in 1-to-1 reference cell mapping.^{15,27} Further, scRNA-seq can provide reference transcriptomic profiles for each cell type that enable the deconvolution of bulk RNA sequencing data using tools such as CIBERSORTx and xCell.³⁹ These computational deconvolution approaches estimate the relative proportions of different cell types in organoids, providing a useful, albeit superficial, layer of cellular composition insight using lower costs bulk methods.

Native Human Gastrointestinal Tissue Reference Data

A growing number of atlas-scale, scRNA-seq datasets of native human tissues are available to support GI organoid benchmarking efforts.^{18,40} These include datasets generated on different longitudinal zones of the healthy GI tract,^{41–43} fetal gut,⁴⁴ neoplasia,^{45,46} and inflammatory diseases^{47–49} (Table 1). When relying on public reference datasets for benchmarking organoids derived from unmatched source tissue, direct comparisons may be affected by inherent biological variability. Available metadata, including tissue origin, developmental stage, and disease status, should be carefully evaluated to identify reference data that most closely matches the biological context of organoids being benchmarked. This is especially important for organoids derived from different GI pathologies, which could be subject to substantial underlying disease heterogeneity. Studies focusing on GI inflammation would benefit from organoid and reference “matching” based on tissue pathophysiology, treatment history, age of disease onset, etc. In addition, analyses of patient-derived colon cancer organoids should consider tumor-specific information such as microsatellite status, mutational profile, and disease subtype. It is important to note that organoid culture conditions can influence proportions of undifferentiated vs differentiated cells and impose selective pressures resulting in expansion of certain subclones over time. Additionally, many organoid systems cannot fully recapitulate native GI tissue due to the absence of environmental cues such as microbiome-derived or stromal components, which are inherently captured in primary tissue datasets. In addition to biological context, technical variation arising from tissue processing, library preparation, sequencing platforms, and computational annotation strategies should be carefully considered, as these factors can influence how scRNA-seq data will align between organoid and native tissue.

Data generated from human specimens are often restricted in metadata availability due to patient consent and confidentiality, which then presents a “missing data” problem. Thus, clearly outlining requirements, limitations, and the depth of available information can substantially improve the reliability of benchmarking efforts. Carefully reporting how foundation datasets were selected and/or

Table 1. Reference Datasets for GI Organoid Benchmarking

In vivo studies	PMID	Donors and patients	Tissues and locations
Cao et al, Science, 2020	33184181	Fetal intestinal donors	Whole intestine
Holloway et al, Cell Stem Cell, 2021	33278341	Fetal intestine donors	Whole intestine
Fawkner et al, Cell, 2021	33406409	Fetal intestine donors	Whole intestine or terminal ileum, proximal colon, distal colon
Yu et al, Cell, 2021	34019796	Fetal and adult intestine donors	Fetal intestine (duodenum, ileum, colon) and adult duodenum
James et al, Nat Immunol, 2020	32066951	Deceased healthy organ donors	Large intestine (cecum, transverse and sigmoid colon) and mesentery lymph nodes
Domínguez Conde et al, Science, 2022	35549406	Deceased healthy organ donors	Small (duodenum, jejunum, ileum) and large intestine (cecum, transverse, sigmoid colon)
Burclaff et al, Cell Mol Gastroenterol Hepatol, 2022	35176508	Deceased healthy organ donors	Small (duodenum, jejunum, ileum) and large intestine (ascending, transverse, descending colon)
Hickey et al, Nature, 2023	37468586	Deceased healthy organ donors	Small and large intestine
Zwick et al, Nat Cell Biol, 2024	38321203	Deceased healthy organ donors	Full-length segmented small intestine
Smillie et al, Cell, 2019	31348891	Patients with CD, UC and healthy donors	Large intestine (right, transverse, left, sigmoid colon and rectum)
Atlasy et al, Nat Commun, 2022	35995787	CeD, non-CeD, GF-diet patients	Duodenal
Huang et al, Cell, 2019	31730855	Pediatric IBD (colitis, UC, CD), control	Large intestine (appendix, cecum, ascending, transverse, descending, sigmoid colon and rectum)
Elmentaite et al, Nature, 2021	34497389	Fetal, pediatric, CD and adult	Small (duodenum, jejunum, ileum) and large intestine (appendix, cecum, ascending, transverse, descending, sigmoid colon and rectum)
Olaloye et al, J Exp Med, 2021	34269788	Fetal, neonatal, NEC patients	Small intestine
Egozi et al, Gut, 2021	37205711	NEC, control (non-injured) patients	Small and large intestine
Lee et al, Nat Genet, 2020	32451460	Adult patients with CRC	Large intestine (cecum, ascending colon, hepatic flexure, sigmoid, rectosigmoid, rectum)
Chen et al, Cell, 2021	34910928	Adult colorectal polyps patients	Large intestine (cecum, ascending, hepatic flexure, transverse, descending, sigmoid, rectum)
Qian et al, Cell Res, 2020	32561858	Adult patients with CRC	Large intestine (cecum, ascending, sigmoid, rectosigmoid)
Zhang et al, Cell, 2020	32302573	Adult patients with CRC	Large intestine (right-side colon and rectum)
Uhlitz et al, EMBO Mol Med, 2021	34409732	Adult patients with CRC	Large intestine (cecum, ascending, transverse, descending, sigmoid colon, rectum)
Che et al, Cell Discov, 2021	34489408	Adult patients with CRC	Large intestine (ascending, transverse, sigmoid colon, upper rectum) + matching liver metastasis and blood
Becker et al, Nat Genet, 2022	35726067	Adult normal, adenomas and CRC patients and deceased donors	Large intestine (ascending, hepatic flexure, transverse, descending, sigmoid colon, rectum)
Joanito et al, Nat Genet, 2022	35773407	Adult colorectal cancer patients	Large intestine (cecum, ascending colon, hepatic flexure, transverse, descending, sigmoid, rectosigmoid, rectum)

Organoid studies	PMID	Donor and patients	Tissue, location and cell line	Sampling	Main cell types	Accession
Uhlitz et al, EMBO Mol Med, 2021	34409732	Patient with CRC	Large intestine (cecum, ascending, transverse, descending, sigmoid colon, rectum)	Adenocarcinoma resection + normal matching tissue	7 patient-derived organoids ± inhibitors	GSE166556
Holloway et al, Cell Stem Cell, 2021	33278341	Fetal intestine donors	Whole intestine	Whole thickness intestine	Fetal enteroids - EGF (3,262), NRG1 (7350) and EGF+NRG1 (2593) treated	E-MTAB-9720
Yu et al, Cell, 2021	34019796	WiCellResearch institute	Human ESC line H9	NC	72,800 - HIO in vitro time course and EGF, NOG, RSPO, NRG1 media conditions and combinations + transplanted	E-MTAB-10268
He et al, Cell Stem Cell, 2022	36002022	Healthy donor patients	Small intestine (ileum and duodenum)	Whole thickness resection	1283 - Patient normal mucosa-derived organoids ± IL22 stimulation	GSE189423
Yang et al, Nat Commun, 2025	39747097	Healthy donor patients	Small intestine	Small intestine biopsies	7595 - Human intestinal organoid grown in TpC conditions	HRA004691 HRA009421
Kirino et al, Com Biol, 2026	41530496	Healthy donor patients	Small intestine (proximal)	Surgical specimens	NC - Day 7 matrigel and day 4 collagen type I grown human intestinal organoids	GSE300603

Online atlases

Human cell atlas	https://www.humancellatlas.org/
Descartes atlas	https://descartes.brotmanbaty.org/bbi/human-gene-expression-during-development/
Human BioMolecular Atlas Program Data Portal	https://portal.hubmapconsortium.org/
Gut cell survey	https://www.gutcellatlas.org/
Human Fetal Gut Atlas	https://simmons-lab.shinyapps.io/FetalAtlasDataPortal/
Human Tumor Atlas Network	https://humantumoratlas.org/

NOTE. This table includes a nonexhaustive list of reference studies for scRNA-seq data. It compiles single-cell atlases from fetal and adult healthy donors or patients with GI-related disorders. A short list of single-cell datasets from organoids is also provided, some extracted from above-mentioned in vivo studies. This table is inspired and has been adapted from “A Roadmap for the Human Gut Cell Atlas.”⁹⁴ CD, Crohn’s disease; CeD, celiac disease; CRC, colorectal cancer; GF, gluten-free; GI, gastrointestinal; HIO, human intestinal organoid; IBD, inflammatory bowel disease; IL22, interleukin22; NC, not communicated; NEC, necrotizing enterocolitis; scRNA-seq, single-cell RNA sequencing; UC, ulcerative colitis.

Box 2. Guiding Principle: Carefully Select In Vivo Reference Data When Benchmarking Organoids by Widely Available Single-Cell RNA Sequencing Methodology

Benchmarking GI organoids using scRNA-seq should prioritize biologically matched reference data, aligned by tissue of origin, developmental stage, and disease context (preferably with matching native tissue reference data), and apply quantitative, transparent comparison frameworks that distinguish true biological deviations from technical or contextual mismatches.

curated for benchmarking organoids will also support reproducibility of organoid benchmarking across studies and laboratories. Large consortia and individual laboratories have made extensive scRNA-seq datasets accessible through interactive portals such as the Broad Institute's Single Cell Portal, the UCSC Cell Browser, CELLxGENE,⁵⁰ and the Human Tumor Atlas Network Data Portal. Tools that enable direct upload and are specifically built for benchmarking de novo organoid scRNA-seq data against reference datasets will further lower computational and technical barriers for the field, whereas the creation of organoid-specific atlases to serve as in vitro references, such as Yu et al²³ or Xu et al,¹⁵ could further standardize benchmarking and support cross-lab reproducibility.

Although such resources provide a useful reference and facilitate organoid-to-tissue comparisons, reliance on unmatched tissues can obscure context-dependent differences and human-to-human variability that potentially confound interpretations of organoid-to-tissue differences. This recognition has increasingly motivated the generation and use of matched organoid-tissue datasets, which provide the most biologically meaningful benchmarks by enabling direct comparison of cell types, transcriptional programs, and functional states between organoids and their in vivo counterparts. Currently, a limited number of studies provide matched in vivo and in vitro organoid data from healthy tissues,¹⁶ developmental contexts,^{5,24} and colon cancer.⁵¹ We envision these efforts will need to expand if the field moves toward the development of benchmarked reference organoid biobanks, in which organoids are rigorously matched to their precise tissue of origin and biological context (Box 2).

Frontiers of Benchmarking

Although gene expression readouts are widely used for unbiased benchmarking of GI organoids, they reflect only 1 facet of functional biology. A more complete assessment requires considering additional layers of cellular activity, including epigenomic and chromatin state, protein abundance and function, cellular morphology and spatial organization, and metabolic behavior (Figure 1). These dimensions capture distinct but interconnected aspects of biology that move us one step closer to "function." Expanding benchmarking to incorporate this broader view will enable a more comprehensive benchmarking of organoid models.

Chromatin and the Epigenome

Although chromatin/epigenomic profiling is typically more costly and time-consuming than RNA profiling, its application to organoid benchmarking is likely to yield substantial biological insights. These include deciphering gene regulatory logic and underlying transcription factor networks and identifying latent enhancers. Adult organoids retain region-specific DNA methylation patterns of their origin tissue, whereas fetal organoids have more capacity to evolve in their epigenomic properties while in culture,^{52,53} as might be expected from studies in developmental biology concerning cellular fate specification, chromatin status, and the rostral-caudal patterning of the gut.⁵⁴ Epigenomic profiling is also critical when mapping the potential function of single nucleotide polymorphisms in human samples. Like RNA, many facets of chromatin biology can be assayed at the single-cell level, facilitating the generation of high-resolution native tissue references and organoid-derived data. These include chromatin accessibility (single-cell assay for transposase-accessible chromatin using sequencing [single-cell ATAC-seq]) and epigenomic profiling (single-cell Cleavage Under Targets and Release Using Nuclease [CUT&RUN], single-cell Cleavage Under Targets and Tagmentation [CUT&TAG], and DNA methylation profiling).⁵⁵⁻⁵⁷ Although application of these methods in the human GI tract has been limited, several studies have used ATAC-seq to profile native tissue and organoids at the single-cell level, which have found that ASC- and PSC-derived organoids display cell type-specific enhancer activity that largely reflects cis-regulatory function observed in native tissue.^{5,58} This demonstrates that organoids preserve key epigenomic features of their tissue of origin, including cell type-specific enhancer dynamics.

When considering specific regions to target for analysis by ATAC-seq or epigenomic profiling, promoter and enhancer regions are primary targets. Both regions vary in their ability to successfully depict the dynamic nature of chromatin status and accessibility in a disease, damage or developmental state. Promoter chromatin tends to be more static compared with enhancer chromatin, as illustrated in a study comparing fetal and adult organoids from mice and in directed differentiation of human induced PSCs.^{53,59} This phenomenon should be considered when benchmarking chromatin status between primary tissues and organoids: enhancer chromatin properties are expected to be more dynamic than promoter chromatin properties and will provide a more detailed and rigorous comparison between cell states or chromatin accessibility status. Enhancers are also more numerous, with some genes receiving input from dozens of different enhancers, and thus benchmarking with enhancer chromatin will be more information-rich than profiling other epigenomic features such as promoter chromatin. Markers of enhancer activity also appear to be more dynamic than epigenomic processes such as 3D chromatin organization.⁵³ Early chromatin benchmarking efforts may therefore benefit the most from a focus on enhancers, with ATAC and single-cell ATAC currently among the easiest entry points due to technical feasibility.

Box 3. Guiding Principle: Consider Biological Variation of Distinct Genomic Features and Impacts of Culture Conditions When Benchmarking Chromatin in Gastrointestinal Organoids

Benchmarking GI organoids using epigenomic methods may benefit from prioritizing enhancers over promoter chromatin or 3D-genome organization. Studies should be mindful of in vivo and ex vivo differences that might influence chromatin states via impacts on epigenomic readers/writers/erasers or through their direct covalent addition to histone tails, including (but not limited to) the presence of microbial metabolites and biomechanical or chemical stimuli.

Finally, enhancers likely contain the lion's share of critical genetic variants affecting the genetic component of prevalent GI-relevant diseases such as inflammatory bowel disease and colorectal cancer.

One emerging consideration in benchmarking organoid epigenomes against native tissues is the extracellular milieu, particularly small molecules produced by the gut microbiome. Butyrate is a known histone deacetylase inhibitor that can impact gene regulation and antagonize proliferation in the gut.⁶⁰ More recent findings also observe covalent addition of microbial metabolites onto histone tails, such as the use of short-chain fatty acids, including butyrate and propionate, as substrates for enzymes catalyzing formation of histone marks (Box 3).⁶¹

Metabolic State and Function

Cellular metabolism is a central determinant of intestinal epithelial identity, function, and disease susceptibility. Along the crypt-villus axis, intestinal stem cells, transit-amplifying cells, and differentiated epithelial lineages exhibit distinct metabolic programs that actively regulate proliferation, differentiation, and stress responses.^{62,63} In vivo, these programs are shaped by steep gradients of oxygen, nutrients, microbial metabolites, and inflammatory signals. Consequently, metabolic state is not merely a downstream readout of cell identity but a dynamic driver of epithelial fate decisions and tissue physiology. Benchmarking GI organoids against primary tissues therefore requires assessing whether organoids capture these state-specific metabolic programs under both homeostatic and disease-relevant conditions.

Although informative, benchmarking by scRNA-seq alone is insufficient to establish metabolic fidelity. Many metabolic pathways are regulated post-transcriptionally through substrate availability, enzyme activity, mitochondrial dynamics, and redox balance. This disconnect is especially apparent when comparing stem-like vs differentiated epithelial populations, where glycolytic, oxidative, and lipid-utilization programs can diverge despite overlapping transcriptional signatures.^{63,64} Thus, transcriptional similarity does not necessarily imply functional metabolic equivalence.

Emerging studies comparing human GI organoids with primary epithelium suggest that organoids broadly

recapitulate core metabolic features of proliferative epithelial states but frequently diverge in ways that reflect culture conditions.^{28,65} Organoids are typically enriched for stem and progenitor populations and maintained in growth factor-rich environments that favor anabolic metabolism and glycolysis. In contrast, differentiated epithelial cells in vivo, such as enterocytes and Paneth cells, engage specialized metabolic programs, leading to metabolic states that are often attenuated by the growth factor-rich conditions used to sustain organoids. These differences become particularly consequential when organoids are used to model disease contexts such as inflammatory bowel disease, colorectal cancer, or enteric infection, where metabolic reprogramming is a core driver of pathology.^{66–68}

Crosstalk with immune cells profoundly shapes epithelial metabolic state through cytokines, lipid exchange, and competition for nutrients. Recent work has demonstrated that disruption of lipid metabolism in tissue-resident immune cells can directly impair epithelial survival and barrier integrity in the human intestine.⁶⁹ Conversely, Chi et al showed that epithelial cystine uptake via SLC7A11 fuels Coenzyme A production that stimulates intraepithelial CD8 $\alpha\beta$ ⁺ T cell-derived interleukin22, thereby promoting intestinal stem cell-mediated regeneration—a metabolic coupling between epithelium and immune cells that standard organoid cultures cannot recapitulate.⁷⁰ Although immune components are largely absent from standard epithelial organoid cultures, these findings underscore a key limitation of organoids as metabolic models and highlight the need to interpret epithelial metabolic benchmarking within the broader tissue ecosystem.

In an ideal scenario, comprehensive metabolic benchmarking would integrate orthogonal approaches, including targeted and untargeted metabolomics, stable isotope tracing to assess pathway flux, mitochondrial functional assays, and spatially resolved metabolic measurements.⁷¹ Matched organoid-tissue pairs derived from the same donor would be particularly powerful, enabling separation of technical divergence from true biological variability.

Recognizing that such approaches may be inaccessible to many laboratories, scalable alternatives can still provide meaningful benchmarks. Targeted metabolite panels, extracellular flux analysis, nutrient-dependence assays, and perturbation-based tests (eg, fatty acid oxidation or glycolysis inhibition) can offer functional insight into whether organoids engage similar metabolic pathways as primary epithelium under defined conditions. When interpreted in the context of epithelial state composition and disease modeling goals, these assays represent practical entry points for metabolic benchmarking.

A persistent challenge remains the limited availability of high-quality metabolic reference datasets for human GI tissue, particularly those integrating cell-type resolution with functional flux across health and disease. Expanding shared metabolic atlases spanning stem and differentiated states, inflammatory conditions, infection, and malignancy will be essential for advancing benchmarking efforts beyond transcriptomics (Box 4).

Box 4. Guiding Principle: Prioritize Study-Relevant Characteristics When Benchmarking Gastrointestinal Organoid Metabolism

Metabolic benchmarking of GI organoids should be state-aware and context-dependent, emphasizing functional metabolic behaviors relevant to the biological question rather than expecting universal metabolic equivalence with primary tissue.

Proteomics and Signaling

Proteomic and phosphoproteomic profiling can provide a functional view of GI biology by directly measuring the molecular effectors and post-translational signaling states that govern epithelial proliferation, differentiation, metabolism, and stress responses. Unlike transcriptomic approaches, proteomics captures post-transcriptional regulation and direct pathway activity, both of which are central to intestinal homeostasis and disease. This is particularly relevant when comparing *in vivo* tissue with *in vitro* organoid systems, where culture conditions and niche factors can rapidly alter protein-level signaling, leading to important changes in cellular phenotype.

Bulk mass spectrometry-based proteomics has been applied to small intestinal and colonic organoids and supports broad concordance with primary tissue in lineage markers and core signaling programs.^{72–74} However, systematic benchmarking of organoid proteomes, particularly phosphoproteomes, against matched primary tissue remains less common than transcriptomic benchmarking. A key conceptual challenge is that cell signaling is inherently dynamic. Bulk, static phosphoproteomic measurements of primary tissue represent heterogeneous mixtures of cell states from multiple discrete cell types that have been shaped by undefined microenvironmental cues. As a result, tissue phosphoproteomics lacks cell-type specific resolution and is often descriptive rather than mechanistic, limiting its utility for defining causal relationships between signaling and function.

Organoids offer a unique opportunity to overcome this limitation. Organoids enable controlled systematic perturbations, such as genetic knockout, ligand stimulation, coculture with heterotypic cell types, or pharmacologic inhibition, that can be directly coupled to phospho-signaling readouts. In this context, changes in phosphorylation can be interpreted as causal responses to defined inputs, rather than passive reflections of an undefined *in vivo* environment. From a benchmarking perspective, this reframes the goal from matching static phosphoproteomic profiles between tissue and organoids to assessing whether organoids recapitulate tissue-relevant signaling logic.

In an idealized setting unconstrained by cost or sample availability, deep quantitative mass spectrometry using multiplexed labeling workflows combined with phosphopeptide enrichment represents the gold standard for benchmarking signaling responses. However, these methods often lack cell type-specific resolution when

Box 5. Guiding Principle: Prioritize Functional Responses When Benchmarking Gastrointestinal Organoid Signaling Networks

Proteomic and phosphoproteomic benchmarking should focus on capturing tissue-relevant signaling logic and perturbation-responsive behaviors, emphasizing functional equivalence over exact molecular abundance.

studying heterocellular systems and can be technically demanding for many laboratories.

Mass cytometry (MC) (also known as cytometry by time of flight) provides a powerful and scalable alternative that aligns naturally with a perturbation-centric benchmarking framework.⁷⁵ MC enables multiplexed measurement of protein expression and phosphorylation states at single-cell resolution, capturing epithelial heterogeneity and signaling responses across thousands of cells. MC has been applied to both native GI tissues using Disaggregation for Intracellular Signaling in Single Epithelial Cells from Tissue (DISSECT) and organoids via Thiol-Reactive Organoid Barcoding *in situ* multiplexing.^{76–78} Recent work used Thiol-Reactive Organoid Barcoding *in situ* MC to construct high-dimensional proteomic and phosphoproteomic ‘phenoscapes’ of human GI organoids across defined microenvironmental perturbations, genetic backgrounds, and patient-derived models, enabling quantitative comparison of cell-state distributions and signaling trajectories.^{79,80}

Where deep mass spectrometry-based proteomics excels in molecular coverage, MC excels in experimental scale, perturbation, and causal interpretability. From a benchmarking standpoint, MC offers several advantages: reusable targeted panels, compatibility with quantitative distance and transport-based metrics, and the ability to compare signaling trajectories rather than static states. A ‘validated’ organoid from a proteomic perspective is therefore not one that exactly matches tissue protein abundance at a single time point, but one that occupies a comparable functional state space and exhibits tissue-consistent signaling responses to defined perturbations. Proteomic benchmarking strategies that embrace the dynamic and causal nature of signaling will be essential if organoid validation is to move beyond descriptive similarity towards mechanistic insight (Box 5).

Morphology, Architecture, and Imaging

The intestinal epithelium exhibits structural adaptations across multiple length scales that amplify key functions, including solute transport and nutrient and water uptake.^{81,82} In humans, millions of millimeter-scale villi extend into the small intestinal lumen, whereas the colon exhibits deep crypts, together providing a ~10-fold increase in surface area. At the single-cell scale, enterocytes and colonocytes extend trillions of micrometer-scale microvilli from their apical surface, increasing functional surface area by an additional ~15-fold. These and other structural features provide a physical basis for optimized epithelial function across the small intestine and colon.

Given the close link between small intestinal and colonic epithelial form and function, structural readouts can serve as valuable and cost-effective readouts for targeted benchmarking. Microscopy remains a primary tool for assessing tissue and cell structure, and the range of imaging technologies is vast in both performance and cost.⁸³ Brightfield, fluorescence, and confocal microscopes are now ubiquitous at research institutions, offering an accessible entry point. Classic histologic stains such as hematoxylin and eosin reveal overall architecture and morphology and can identify basic differentiation of small intestinal organoids through Paneth cells by their granules. Periodic acid–Schiff staining highlights mucin-rich goblet cells in both small intestinal and colonic organoids. Although these stains alone do not indicate *in vivo* levels of functionality, the presence of granules or mucins reflects progress toward a functional GI organoid.

Microscopy can also assess how well organoids recapitulate *in vivo* differentiation programs. Cells originating in stem cell-containing crypts adopt compartment-specific morphologies as they migrate toward the villus tip or colonic surface, many of which are recapitulated in 3D organoid cultures.^{80–82} For example, columnar epithelial cells in organoids expand their apical domains relative to crypt-based cells.⁸⁴ This apical area expansion can be measured in GI organoids using fix-and-stain methods with junctional markers, confocal imaging, and cell segmentation in free software such as FIJI and compared with native GI tissue.⁸⁵ Similarly, apical enrichment of F-actin, detected with phalloidin, marks brush border formation in enterocytes and colonocytes, providing a quantitative readout of microvillus assembly.^{83,86,87}

Other measurements of organoid differentiation, such as cell height, proliferation, or polarity, can also be assessed by microscopy. Cell height in 3D organoids or monolayers can be compared with *in vivo* measurements of cell heights from small intestine villus or colonic surfaces.^{88,89} Additionally, differentiated GI organoids from healthy tissue typically show lower proliferation, reflecting the nonproliferative nature of healthy differentiated gut epithelial cells.^{89–92} Many 3D organoids also shed cells into the lumen, similar to the villus tip or colonic surface *in vivo*.^{93,94} These features should still be interpreted in the context of the tissue of origin or regional specification, which may corroborate variation attributed to matching GI tissue.

Modern microscope systems offer the potential for more complex, unbiased benchmarking with automation for multiwell formats, and deep learning/artificial intelligence–based analysis that can simplify extraction of morphometric parameters.^{85,87} Simultaneously, emerging light-sheet microscopy approaches extend this paradigm by enabling live, multiscale 3D imaging of entire organoids over time, generating digital organoid atlases that integrate geometry, cell morphology, and lumen structure.⁸⁶ These dynamic imaging capabilities are beginning to reveal cellular behaviors and structural changes that are difficult to capture in native tissue, highlighting both new opportunities and challenges for benchmarking GI organoids against *in vivo* counterparts.

Box 6. Guiding Principle: Scale Morphologic Benchmarking to Targeted or Unbiased Analysis, Dependent on Study-Specific Goals

Morphologic benchmarking should assess whether organoids adopt native-like cell type localization, epithelial geometry, apical specialization, and cell movement, capturing differentiation-dependent architectural features rather than replication of every tissue dimension.

These morphologic and staining workflows provide both simple and complex examples of structural benchmarking assays that should be feasible at most biomedical research institutions. Although framed around 3D organoid cultures, newer 2D monolayer systems enable other microscopy modalities that hold the potential to reveal key dynamic events in intestinal epithelial biology (eg, the crypt–villus transition or collective migration along the crypt–villus axis).^{95,96} Adventurous researchers will find no shortage of advanced technologies for extending organoid benchmarking toward dynamic changes over time or below the diffraction limit with super-resolution microscopy (Box 6).^{86,97}

Improving the Functional Accuracy of Gastrointestinal Organoid Models Through Benchmarking

In addition to the evaluation of *in vitro* systems, benchmarking can guide functional optimization of organoids. Comparisons between GI organoids and matched *in vivo* tissues have revealed missing cell types, skewed differentiation trajectories, or disrupted signaling programs. Organoids also show inherent variability due to patient origin, which can be further influenced by alterations in cellular composition and gene expression associated with passage number and prolonged culture.^{40,98} Many ASC-derived organoids remain largely undifferentiated under standard culture conditions, lacking exposure to microbiome or stromal components, therefore complicating functional benchmarking and assessment of physiological relevance.²⁹ Some of these deviations from native GI tissue can be corrected through targeted culture modifications including growth factors, small molecules, or coculture strategies. Fujii et al compared the *in vivo* intestinal niche with 3D organoid composition, showing that altering components of the epidermal growth factor receptor niche enabled long-term culture with greater cellular diversity.²⁸ More recently, Yang et al described a ‘tunable’ model for human GI organoids, where a combination of 3 small molecules increased cellular diversity while preserving stem cell function.¹⁶ Although small-molecule and growth factor optimization are necessary to improve cell function in an isolated state, achieving full physiological relevance often requires incorporating additional cell types found in native tissue.

Coculturing organoids with stromal or immune cells has further improved physiological relevance. Organoids cultured in monolayers with macrophages possess

Box 7. Guiding Principle: Apply Benchmarking to Improve Biological and Functional Relevance of Gastrointestinal Organoids

Functional benchmarking highlights how organoid systems reflect tissue-relevant behaviors and responses and indicates avenues where further refinement and validation are needed as organoid models become more complex.

enhanced structural features, including increased epithelial cell height and secretory response polarization to more native-like behavior.⁹⁹ Even in more fetal-like conditions with PSC-derived organoids, cocultured macrophages exhibit features closer to tissue-resident macrophages.¹⁰⁰ Importantly, PSC-derived organoids can spontaneously generate mesenchymal components, including smooth muscle, vasculature, and neurons, reflecting their intrinsic developmental capacity without requiring coculture while contributing to increased structural and cellular complexity.^{10,32,101,102} Complementing these observations, coculturing organoids with matched fibroblasts from tumors improves functional fidelity, restoring organoids to more accurately reflect the subtype and characteristics of the tumor of origin.¹⁰³ Together, these emerging changes to GI organoid platforms demonstrate that integrating benchmarking into research pipelines can systematically enhance questions we can ask and answer with GI organoid models.

Bioengineered intestinal tissues incorporating scaffold-guided architecture alongside stromal and immune cell components represent a significant departure from traditional epithelium-only, closed organoid systems.²⁷ These organ-on-chip systems can often establish spatially defined growth factor gradients, enabling morphogen patterning and differentiation programs that more closely resemble in vivo tissue organization.^{27,104,105} Establishing benchmarking standards for these complex models will require new metrics that capture tissue-level organization, multicellular interactions, lumen architecture and flow, peristalsis, and response dynamics to infection. Functional benchmarking is important, even for studies not explicitly focused on cell or gut function, as understanding the behavior and responses of the system compared with native GI tissue is essential for interpreting experimental results. The extent or type of functional benchmarking that is necessary will vary, with experimental and biological context dictating the functional requirements of the organoid system. As the field advances, benchmarking frameworks must evolve alongside increasingly sophisticated model systems beyond what current approaches assess (Box 7).

Conclusions

Benchmarking GI organoids requires substantial effort. Foundation datasets spanning multiple tissues and derived from wide-ranging donor and patient cohorts are powerful resources but can also be challenging to interpret. Whether

harmonizing existing datasets and/or relying on newly generated reference data, benchmarking strategies benefit from being tailored to the specific objectives of each study. Meaningful benchmarking is also reliant on reproducible practices in organoid research. As discussed in the first article of this series, “*Guiding Principles: Reporting Standards for Gastrointestinal Organoid Research*,”¹⁰⁶ standard reporting is the initial step toward robust benchmarking practices. Regardless of whether researchers follow reporting standards laid out in this *Guiding Principles* series or develop their own, careful documentation of organoid handling and quality control—including tissue of origin, culture system, passage number, and media conditions—is critical to ensure the meaningfulness of benchmarking efforts across the field.

As organoid systems, data, and questions become increasingly sophisticated, benchmarking frameworks must evolve in parallel, integrating structural, functional, and multi-omic readouts to ensure that organoid models faithfully capture human in vivo biology. Achieving this will require a collaborative, community-wide effort. We invite researchers to share organoid datasets in public repositories, participate in cross-laboratory benchmarking initiatives, and engage in ongoing dialogue to refine standards that are both rigorous and broadly accessible. At the same time, key stakeholders, including funding bodies, journal editors, and reviewers, must actively recognize and incentivize these efforts, supporting both individual laboratories and consortia in the development, validation, and dissemination of benchmarked organoid systems. Benchmarking is a long-term investment; it strengthens individual expertise, advances the collective rigor of the scientific community, and deepens our understanding of human biology. By committing to this effort, we can set enduring standards for reproducibility and fidelity, ultimately enabling discoveries that advance human health and clinical translation.

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Review Panel

Kathryn E. Hamilton, PhD, Rachel K. Zwick, PhD, Jorge Orlando Munera, PhD, Robert Barrett, PhD, Brooke R. Druliner, PhD.

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