Research Square

Preprints are preliminary reports that have not undergone peer review.
They should not be considered conclusive, used to inform clinical practice,
or referenced by the media as validated information.

Generating Colonic Organoids from Cryopreserved Human Mucosa: Feasible, Yet Still Challenging

Romain Dautreppe
Université de Toulouse, INSERM, INRAE, ENVT, IRSD
David Sagnat
Université de Toulouse, INSERM, INRAE, ENVT, IRSD
Laurence Picque-Lasorsa
IRCM (Montpellier Cancer Research Institute), INSERM, ICM, University of Montpellier
Céline Deraison
Université de Toulouse, INSERM, INRAE, ENVT, IRSD
Nathalie Vergnolle
Université de Toulouse, INSERM, INRAE, ENVT, IRSD
Fabien Granier
FGHI
Thibault Mazard
IRCM (Montpellier Cancer Research Institute), INSERM, ICM, University of Montpellier
Corinne Prévostel
IRCM (Montpellier Cancer Research Institute), INSERM, ICM, University of Montpellier
Claire Racaud-Sultan
claire.racaud@inserm.fr

Université de Toulouse, INSERM, INRAE, ENVT, IRSD

Research Article

Keywords: Organoids, Frozen mucosa, Colorectal cancer, Organoid culture media

Posted Date: November 24th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11377225/v1

License: This work is licensed under a Creative Commons Attribution 4.0 International License.
Read Full License

Additional Declarations: No competing interests reported.

Generating Colonic Organoids from Cryopreserved Human Mucosa: Feasible, Yet Still Challenging

Romain Dautreppe[a], David Sagnat[a], Laurence Picque-Lasorsa[b,c], Céline Deraison[a], Nathalie Vergnolle[a], Fabien Granier[d], Thibault Mazard[b,c], Corinne Prévostel[b], Claire Racaud-Sultan[a]

[a]Université de Toulouse, INSERM, INRAE, ENVT, IRSD, Toulouse, France.

[b] IRCM (Montpellier Cancer Research Institute), INSERM, ICM, University of Montpellier, 34298 Montpellier, CEDEX 5, France.

[c]Medical Oncology Department, ICM, University of Montpellier, CEDEX 5, 34298 Montpellier, France.

[d]FGHI, 165 Rue Denis Papin, 34570 Montarnaud, France.

Corresponding author: Claire Racaud-Sultan, MD, PhD, IRSD Institut de Recherche en Santé Digestive, UMR 1220/1416, CHU Purpan, Place du Docteur Baylac, CS60039, 31024 Toulouse cedex 3, France, Tel: 33-562746142, Fax: 33-562744558, e-mail :claire.racaud@inserm.fr

Running title: Organoid production from frozen mucosa

ABSTRACT

Background: Colorectal cancer organoids serve as powerful ex vivo models that recapitulate tumor heterogeneity, enabling mechanistic studies and personalized drug screening. However, the logistical challenges of transporting fresh human tissue often limit their clinical and research implementation. In this study, we evaluate the feasibility of generating organoids from cryopreserved human intestinal mucosa. **Methods**: Paired healthy and cancerous mucosal samples from 23 patients with colorectal cancer were cryopreserved and subsequently processed for organoid culture using various media formulations. **Results**: Our findings identify critical parameters for successful organoid recovery: 1/ a cryopreservation duration of ≤ 4 weeks; 2/ a significantly higher recovery rate from left colonic tissue and/or early-stage cancer, regardless of the culture medium used. While the current success rate was 50%, a comparative analysis with existing literature suggests that optimizing cryopreservation and epithelial dissociation media could further enhance efficiency. **Conclusions**: Generating organoids from frozen mucosa is a viable strategy for clinical, pre-clinical and basic research applications. Further studies involving larger cohorts are warranted to validate these findings and refine the protocol for maximum yield.

Keywords: Organoids, Frozen mucosa, Colorectal cancer, Organoid culture media

Background

Patient-Derived Tumor Organoids (PDTOs) have emerged as powerful tools for advancing colorectal cancer (CRC) therapy, with extensive validation in both clinical and preclinical studies [1,2].

However, their broader limitation is constrained by the limited time window between surgery and organoid processing, which is critical for maintaining *ex vivo* cell viability. While cryopreservation could overcome this limitation, published data on generating organoids from cryopreserved samples remain limited [3,4].

Here, we share our experience in deriving organoids from 23 human colorectal tissues (paired healthy and tumor mucosa) collected and cryopreserved at the Cancer Research Institute of Montpellier (ICM) between 2024 and 2025 and processed for organoid culture at the Digestive Health Research Institute of Toulouse (IRSD), two Institutes located 250 km apart.

Our findings highlight the feasibility of this approach while underscoring the persistent technical challenges and its potential to expand patient eligibility for organoid-based applications, including retrospective and future studies.

Methods

Study Population

Approved by the ICM's "Comité de Recherche Translationnelle" (ICM-CORT-2022-19), our study prospectively enrolled 23 untreated CRC patients after obtaining informed consent. Key diagnostic details including age, gender, tumor site, stage and molecular status (mismatch repair, MMR and/or microsatellite instability/stability, MSI/MSS, *RAS* and *BRAF* mutations) when available were provided to the organoid production team (Table I).

Table I: Clinical and biological profiles of the patient cohort undergoing organoid culture. Samples consist of paired and cryopreserved healthy (H) and colorectal cancer (CRC) mucosa. The median age of the included patients is 69 years.

Patient N°	Gender	Age	Tumor site	Histologic subtype Grade	Molecular status	Stage	Time in cryopreservation	Organoid production	Organoid biobanking
1	F	≥69	Caecum	Adenocarcinoma NOS (mucinous component=20%) High grade	*KRAS* G11K mutation *BRAF* wild-type dMMR/MSI	II (pT3N0)	55 days	H: **Success** CRC: **Failure**	Yes
2	M	≥69	Ascending colon	Adenocarcinoma NOS Low grade	*KRAS* A146V mutation *BRAF* wild-type pMMR/PCR MSI not done	II (pT3N0)	39 days	H: **Success** CRC: **Failure**	Yes
3	M	<69	Sigmoid	Adenocarcinoma NOS Low grade	*RAS* and *BRAF* not done pMMR/PCR MSI not done	III (pT4N1b)	34 days	H: **Success** CRC: **Success**	Yes
4	F	<69	Rectosigmoid	Adenocarcinoma NOS Low grade	*RAS* and *BRAF* not done pMMR/PCR MSI not done	II (pT3N0)	27 days	H: **Success** CRC: **Success**	Yes
5	M	≥69	Left colic angle	Serrated adenocarcinoma Low grade	*KRAS* G12C mutation *BRAF* wild-type pMMR/PCR MSI not done	II (pT4N0)	60 days	H: **Success** CRC: **Failure**	Yes
6	M	≥69	Caecum	Mucinous adenocarcinoma High grade	*KRAS* G12V mutation *BRAF* wild-type pMMR/PCR MSI not done	IV (pT3N2aM1c)	53 days	H: **Failure** CRC: **Success**	Yes
7	M	≥69	Caecum	Medullary carcinoma NA grade	*RAS* wild-type *BRAF* V600E mutation dMMR/MSI	II (pT3N0)	46 days	H: **Success** CRC: **Failure**	Yes
8	F	<69	Ascending colon	Adenocarcinoma NOS (mucinous component=35%) High grade	*RAS* wild-type *BRAF* V600E mutation dMMR/MSI	II (pT3N0)	29 days	H: **Success** CRC: **Failure**	Yes

12	M	<69	Sigmoid	Adenocarcinoma NOS Low grade	*RAS* and *BRAF* not done pMMR/PCR MSI not done	II (pT4N0)	42 days	H: **Success** CRC: **Failure**	Yes
13	F	≥69	Caecum	Adenocarcinoma NOS (mucinous component=25%) High grade	*KRAS* G13D mutation *BRAF* wild-type pMMR/PCR MSI not done	III (pT4N1a)	37 days	H: **Failure** CRC: **Success**	Yes
14	M	<69	Right colic angle	Micropapillary adenocarcinoma High grade	*NRAS* G11K mutation *BRAF* wild-type pMMR/PCR MSI not done	III (pT3N2b)	35 days	H: **Success** CRC: **Failure**	Yes
15	M	≥69	Right colic angle	Adenocarcinoma NOS (mucinous component=35%) High grade	*RAS* and *BRAF* not done dMMR/PCR MSI not done	III (pT4N2b)	34 days	H: **Failure** CRC: **Failure**	No
16	M	≥69	Ascending colon	Adenocarcinoma NOS Low grade	*RAS* and *BRAF* not done pMMR/PCR MSI not done	III (pT4N2a)	21 days	H: **Failure** CRC: **Failure**	No
17	M	≥69	Caecum	Adenocarcinoma NOS Low grade	*RAS* and *BRAF* not done pMMR/PCR MSI not done	I (pT2N0)	21 days	H: **Failure** CRC: **Success**	Yes
18	F	≥69	Ascending colon	Adenocarcinoma NOS Low grade	*RAS* and *BRAF* not done dMMR/MSI	III (pT3N1a)	13 days	H: **Success** CRC: **Success**	Yes
19	F	<69	Right colic angle	Adenocarcinoma NOS Low grade	*RAS* and *BRAF* not done pMMR/PCR MSI not done	III (pT4aN1b)	63 days	H: **Failure** CRC: **Failure**	No
20	F	≥69	Right colic angle	Serrated adenocarcinoma Low grade	*KRAS* G13C mutation *BRAF* wild-type pMMR/MSS	II (pT3N0)	43 days	H: **Failure** CRC: **Failure**	No
21	F	<69	Caecum	Adenocarcinoma NOS (mucinous component=40%)	*KRAS* G13D mutation *BRAF* wild-type	IV (pT3N2b)	21 days	H: **Failure** CRC: **Failure**	No

Cryopreservation of Mucosa

Paired tumoral and adjacent healthy tissues (0.5 to 1 cm^3 each per patient) were collected during primary tumor surgeries at the Institut du Cancer de Montpellier (ICM, collection CRB-ICM N°BB-0033-00059) between 2024 and 2025 and immediately preserved in Custodiol™ (Solution HTK, Ref. 1722171 Dr. Franz Köhler Chemie GmbH, Bensheim, Germany). Within hours post-surgery, the tissue fragments in Custodiol were processed to isolate the mucosa. After three washes in PBS, the mucosa was dissected from the surrounding tissue and cut in 2 or 3 pieces of approximately 5 mm^2 each. These mucosal pieces were then preserved in advanced Dulbecco's modified Eagle medium/F12 (DMEM F12 *plus* Glutamax, Ref. 11514436 Fisher Scientific Illkirch, France) supplemented with 10% fetal bovine serum (FBS, Ref. F7524 Sigma-Aldrich, Saint-Quentin-Fallavier, France) and 10% dimethyl sulfoxide (DMSO, Ref. D2660 Sigma-

Aldrich) at -80°C.[3] Once samples from 2 to 4 patients were collected, they were transported in dry ice (within ≤ 24 hours) to the IRSD organoid facility in Toulouse.

Production and Preservation of Organoids

The protocol for frozen mucosal samples was adapted based on our prior experience with fresh tissue [5-7]. Upon arrival at the organoid facility in Toulouse, samples were immediately thawed using one of two methods: 1- three 5-minute washes in phosphate buffer saline (PBS) supplemented with 43.4 mM sucrose, 54.9 mM sorbitol, 3 mM dithiothreitol, and 100 U/mL penicillin-streptomycin, or 2- direct thawing in advanced DMEM/F12 medium supplemented with 10% FBS.

Crypts were then isolated by subjecting mucosal samples to 15 minutes of moderate agitation in PBS with 8 mM ethylenediaminetetraacetic acid (EDTA), 10 µM Y27632 (Ref. Y0503 Sigma-Aldrich), 1 mG/mL primocin, followed by two 2-minute rounds vigorous manual agitation in the same buffer without EDTA. For selected experiments, crypt isolation was performed in the presence of 500 µL TrypLE™ (Ref. 10043382, Fisher Scientific). Isolated crypts (approximately 1000 crypts derived from three mucosal pieces) were then centrifuged at 40x *g* for 5 minutes at 4°C. The supernatant was discarded, and the crypt-containing pellet was resuspended in Matrigel™ (Ref. 356231, Corning, Amsterdam, the Netherlands). Aliquots of 20 µL Matrigel™ containing 100 crypts were deposited per well in a 48-well plate (Ref. 677180, Greiner Bio-One, Les Ulis, France). After 10 minutes of gel polymerization at 37°C, various culture media were tested in duplicate wells (Table II):

-Standard medium containing advanced DMEMF12 supplemented with 50% Wnt3a-conditioned medium and 1 µg/mL R-spondin 1, with or without a p38 MAPK inhibitor;

-Standard medium containing advanced DMEMF12 supplemented with 5% Wnt3a-conditioned medium and 1 µg/mL R-spondin 1, with or without a p38 MAPK inhibitor;

-Standard medium containing advanced DMEMF12 supplemented with 50% Wnt3a-conditioned medium without R-spondin 1, with or without a p38 MAPK inhibitor;

-Alternative medium containing RPMI 1640 (Ref. 12633-012, Fisher Scientific) supplemented with 50% Wnt3a-conditioned medium and 10% FBS, 1 µg/mL R-spondin 1, 10% FBS, with or without a p38 inhibitor;

-Alternative medium containing RPMI 1640 supplemented with 5% Wnt3a-conditioned medium and 10% FBS, 1 µg/mL R-spondin 1, 10% FBS, with or without a p38 MAPK inhibitor.

For organoids derived from healthy mucosa, culture was exclusively performed using standard medium containing advanced DMEMF12 supplemented with 50% Wnt3a-conditioned medium, R-spondin 1, and with p38 inhibitor. This standard medium has been largely validated by us and others for the culture of organoids from human healthy mucosa [5-7]. In contrast, all tested media were evaluated for organoid cultures derived from tumoral samples given their heterogeneity and their unpredictable responses [8].

Table II: Composition of organoid culture media. The TGFβ-R inhibitor (A-8301) and the Rho kinase inhibitor (Y27632) were supplemented only during the first 48 hours of culture, except for the normal samples in P0, which were maintained with Y27632 until passage.

	Standard medium 50% Wnt3a	Standard medium 5% Wnt3a	Standard medium w/o R-spondin	Alternative medium 50% Wnt3a	Alternative medium 5% Wnt3a	Reference Supplier
Advanced DMEM F12	+	+	+	-	-	11514436 Fisher Scientific
RPMI 1640	-	-	-	+	+	12633012 Fisher Scientific
Wnt3a-conditioned medium	50%	5%	50%	50%	5%	CRL2648 ATCC
R-spondin 1 (1 µG/mL)	+	+	-	+	+	4645RS100CF Fisher Scientific
FBS (10%)	-	-	-	+	+	F7524 Sigma-Aldrich
p38 inhibitor (SB202190 10 µM)	±	±	±	±	±	57067 Sigma-Aldrich
Hepes (10 mM)	+	+	+	-	-	15630056 Fisher Scientific
Glutamax (2 mM)	+	+	+	-	-	A1286001 Fisher Scientific
N2 (1/100)	+	+	+	+	+	17502048 Fisher Scientific
B27 w/o Vit. A (1/50)	+	+	+	+	+	1287010 Fisher Scientific
EGF (50 nG/mL)	+	+	+	+	+	PHG0314 Fisher Scientific
Noggin (100 nG/mL)	+	+	+	+	+	12010C Fisher Scientific
PGE2	+	+	+	+	+	P0409

After 8 to 10 days of culture at 37°C in a 5% CO_2 atmosphere with medium changes every 2 days, the organoids were dissociated into single cells. This was achieved by incubation with TrypLE™ at 37°C for 10 minutes under agitation, followed by mechanical dissociation by pipetting. The resulting single-cell suspension was then resuspended in advanced DMEMF12 supplemented with 2 mM Glutamax, 10 mM Hepes, 10 µM Y27632 and 10% FBS and centrifuged at 400x *g* for 5 minutes at 4°C. The pellet was subsequently resuspended in Matrigel™ and the cell- Matrigel™ mixture was deposited into culture wells for the continuation and expansion of the organoid culture. After one or two passages, Matrigel-embedded organoids were recovered in Synth-a-Freeze™ (Ref. 12053479, Fisher Scientific) on ice and then frozen at -80°C for long-term conservation in liquid nitrogen.

Statistical Methods

Fisher' exact test (GraphPad Prism 10, Boston, MA, USA) was used to measure the significance of repartitions of clinical and biological parameters of patients between success and failure groups to produce organoids.

Results

Characteristics of the Cohort

Twenty-three patients were included with a median age of 69 years [43-92] and an equal gender distribution (Table I).

Primary tumor site was right colon (from caecum to transverse colon) in a majority of the cases (70%) and the histologic type was 43% adenocarcinoma vs others (mucinous component, …). Tumor stage distribution was I (8.5%)/ II (48%)/ III (35%) and IV (8.5%).

30% exhibited a dMMR and/or MSI status and of the 11 tumors tested, 8 had a *RAS* mutation and 2 a *BRAF*^{V600E} mutation.

Challenges in Organoids Production from Frozen Mucosa

Under our experimental conditions, the organoid production rate from frozen mucosa was 48%. The success rate for frozen colorectal cancer tissue was 39% compared to 56% for frozen healthy tissue. In 41% of the failed cultures, the inability to generate organoids occurred in both healthy and tumoral tissues from the same patient. This suggests that success is governed by patient-specific factors (e.g, tumor site/histologic type/stage, molecular status…), sample processing parameters (cryopreservation duration/condition, thawing/culture conditions….) or a combination of both. Morphologically, organoids derived from healthy mucosa developed as cysts whereas those from tumoral mucosa exhibited a mixed morphology comprising both spheroids and cysts (Fig. 1).

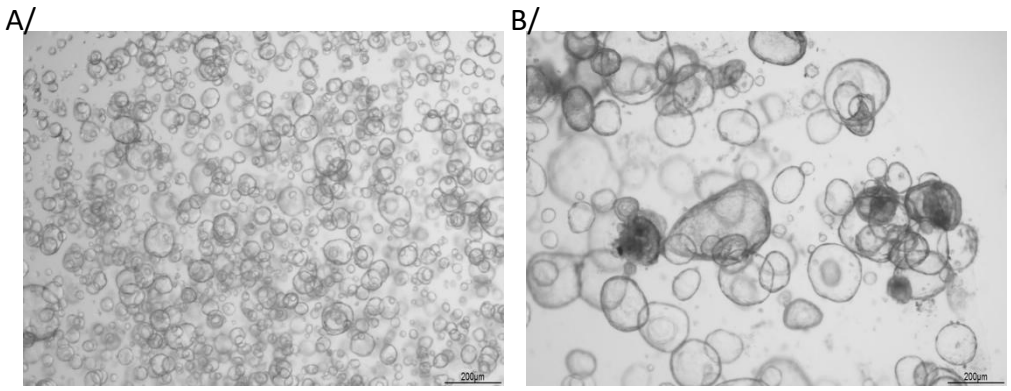


Figure 1: Morphological features of patient-derived organoids generated from crypts isolated from healthy and CRC mucosa. Healthy mucosa organoids grew exclusively as cysts (A) whereas tumoral mucosa organoids exhibited both spheroid and cystic

morphologies (B). Organoids were cultured in the standard medium supplemented with 50% Wnt3a (Table II). Images were acquired at day 10 (passage 1) of culture using a Nikon TS2 eclipse microscope (4x, brightfield).

In our study, mucosal samples were preserved in advanced DMEMF12 supplemented with 10% FBS and 10% DMSO at -80°C for periods ranging from one week to two months before being thawed for organoid production (Table I). However as shown in Fig. 2 the failure rate of organoid cultures derived from frozen colonic tissues increased significantly after 6 weeks for healthy tissue and 4 weeks for tumoral tissue. These findings suggest that shortening the cryopreservation period under these experimental conditions may enhance organoid viability.

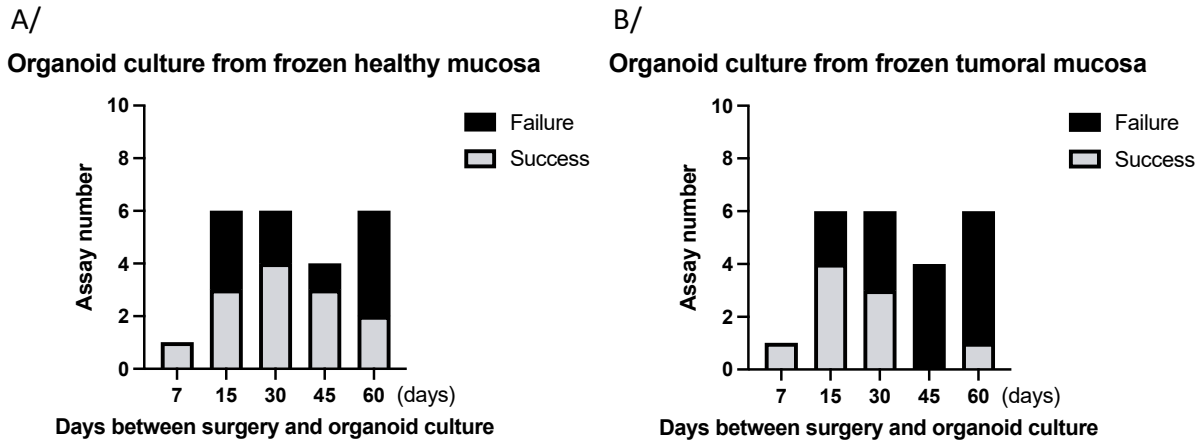


Figure 2: Effect of cryopreservation duration on the establishment of patient-derived colonic organoids. Colonic crypts were isolated from frozen healthy or tumoral mucosa of patients (n=23) and cultured as described in the Methods section. Data represent the

success and failure rates of organoid recovery expressed as the number of assays relative to the duration of mucosal cryopreservation (days).

To address the heterogeneity of CRC samples, we tested various culture media to enhance organoids production yields (Table II). These included DMEMF12 or RPMI1640 (selected based on metabolic status, [9]), supplemented with variable concentrations of Wnt3a-conditioned medium and with or without the p38 inhibitor SB202190 to promote CRC proliferation [8], and with or without R-spondin 1 to enhance the PI3-kinase pathway [10,11].

Our aim was to generate organoids from both healthy and tumoral colic tissues under highly controlled conditions to enable comparative pharmacological analyses. Given that the failure rate of organoid cultures – from healthy and tumoral tissues per patient- became prominent after 1 month of cryopreservation (Fig. 4), whatever the protocol used for crypt isolation (EDTA or TrypLE, see Methods section), and the unpredictable responses of CRC tissue to the supplementing factors [8], we compared organoid production across the different culture media using mucosal samples from 14 patients, all cryopreserved around 4 weeks (Patients N°2,3,4,8,9,10,13,14,15,16,17,18,21,23, Table I).

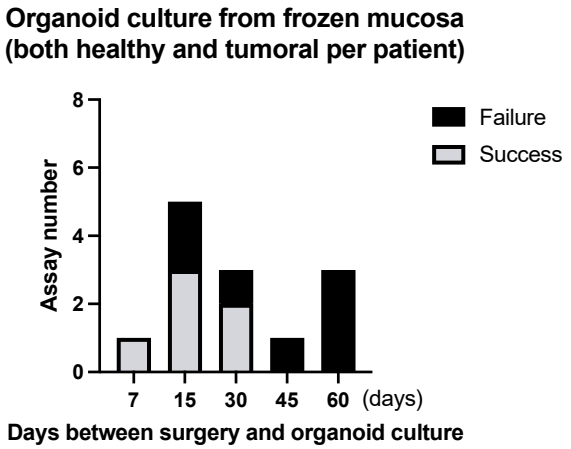


Figure 4: Impact of colonic mucosa cryopreservation duration on the success and failure rates of the production of organoids generated from both healthy and tumoral mucosa, for each patient. Colonic crypts isolated from frozen healthy and tumoral mucosa of each patient (n=13) were cultured as organoids as described in the Methods section. The success or failure of organoid cultures are reported as assay numbers per duration in days of the mucosa cryopreservation.

Between 7 and 13 samples have been tested in each culture condition (Table II) with a rate of success in organoid production from 43% to 54% when media were supplemented with 5% or 50% Wnt3a, respectively. The failure or success of organoid culture was independent of the culture condition tested – either all media (Table II) succeeded or failed for a given sample (not shown). Healthy and tumoral tissues did not differ in the distribution between success and failure groups of organoid culture, and no significant differences were observed in gender, age, and MSI distribution (Table III). However, for tumors and their normal adjacent tissues [12], the tumor site was

200 significantly correlated with outcomes and the tumor stage showed a similar trend (Table III): organoid cultures derived from left colon
201 and early-stage CRC showed higher success rates for amplification and then biobanking while those from the right colon and advanced-
202 stage CRC were more challenging to maintain and expand.

203 **Table III: Correlation between clinical/biological parameters and organoid establishment success.** Analysis of n=14 patients
204 (median age : 69 years) whose colonic mucosa (paired healthy and tumoral) were processed for organoid culture within 40 days of
205 cryopreservation. Success and failure rates are analysed in relation to clinical and biological variables.

	Healthy organoid cultures (n=14)		Fisher's exact test	**Tumoral organoid cultures (n=14)**		Fisher's exact test	**Healthy/Tumoral organoid cultures (n=28)**		Fisher's exact test
	Success (n=9)	**Failure (n=5)**		**Success (n=8)**	**Failure (n=6)**		**Success** 9/8	**Failure** 5/6	>0.9
Sex (M/F)	5/4	3/2	>0.9	4/4	4/2	0.63	9/8	7/4	0.7
Age (<69y/≥69y)	6/3	1/4	0.26	4/4	3/3	>0.9	10/7	4/7	0.44
Primary tumor side (Left/Right)	4/5	0/5	0.22	4/4	0/6	0.08	8/9	0/11	<0.01
Stage (I-II/III-IV)	6/3	1/4	0.26	5/3	2/4	0.59	11/6	3/8	0.12

pMMR/dMMR	6/3	4/1	>0.9	6/2	4/2	>0.9	12/5	8/3	>0.9

206

207

208 **Discussion**

209 The production of organoids from frozen mucosal samples showed markedly lower efficiency compared to fresh tissue processing. While
210 both the literature [13], and our previous experience, indicate an ~80% success rate for organoids derived from fresh healthy or tumoral
211 tissues, our yield from frozen samples was only 48%. Other studies have reported higher success rates: 100% success for organoids from
212 frozen healthy colonic tissues (n=13 patients, [3]), and 94% success for organoids from frozen tumoral tissues (n=16 patients, [4]).
213 Consistent with our observations, these studies noted that frozen-derived healthy organoids typically exhibit cystic morphology and
214 diminished growth kinetics compared to their fresh counterparts, whereas frozen-derived tumoral organoids display a heterogeneous
215 morphology composed of both cysts and spheroids.

216 Optimizing the cryopreservation and dissociation protocols may be key to enhancing the yield from frozen colonic tissues. Our findings
217 suggest that minimizing the cryopreservation interval could be beneficial. For instance, Tsai *et al*. achieved a 100% of success rate using
218 a protocol similar to ours (cryopreservation, epithelial cells isolation, and standard culture media) but their mucosal samples were stored
219 for only 1 to 4 weeks post-surgery (n=3 patients, [3]). This supports our hypothesis that shortening the cryopreservation duration to
220 within 1 month may significantly improve organoid viability under these experimental conditions. Conversely, Chen *et al*. reported a

221 94% of success rate for organoid production from CRC samples cryopreserved for 4 to 43 months after surgery (median 7.5 months;
222 n=16 patients, [4]). Their superior performance might be attributed to their specific cryopreservation in a classical medium (90%
223 FBS/10% DMSO) followed by a transfer to liquid nitrogen for long-term storage, and a robust tissue dissociation step using
224 collagenase/hyaluronidase at 37°C for 1-2 hours before organoid culture. It remains to be determined whether adopting such
225 cryopreservation and dissociation protocols would enhance the production rates of organoids for both healthy and tumoral tissues in our
226 setting.
227 Furthermore, we observed enhanced organoid recovery from left-sided colonic tissue and/or early-stage cancers, regardless of the culture
228 medium used. Interestingly, the single failure reported in the cohort of 16 CRC patients reported by Chen *et al*. involves a 65-year-old
229 female with stage IV CRC localized in the right colon [4]. Although the clinical and biological distinctions between right- and left-sided
230 CRC are well documented [14], their impact on organoid establishment from fresh tissues remains largely unexplored. Nevertheless,
231 these inherent biological differences may contribute to the increased resilience of left-sided CRC to the transition from frozen mucosa
232 to organoid culture.
233
234 **Conclusions**
235 The ability to generate high-quality organoids from cryopreserved mucosal samples holds significant promise for clinical, preclinical,
236 and basic research applications. Our findings suggest that optimizing the establishment efficiency requires a multi-faceted approach:
237 implementing a specialized cryopreservation medium (e.g., 90% FBS/10% DMSO) coupled with long-term liquid nitrogen storage,

refining enzymatic dissociation protocols and tailored culture media to address the inherent genetic and epigenetic heterogeneity of CRC, including its anatomical localization [9]. Future studies involving larger, more diverse patient cohorts will be essential to validate these strategies and standardize protocols for clinical translation.

List of abbreviations

CRC, colorectal cancer; MSI, microsatellite instability; MMR, mismatch repair.

Declarations

Ethics approval and consent to participate

Human colon tissues were obtained at the Medical Oncology Department of ICM (Institut du Cancer de Montpellier), University of Montpellier. All patients included in this study were orally informed of the purpose and contents of the study before consenting. This study was approved by the ICM's "Comité de Recherche Translationnelle" (Approval N° ICM-CORT-2022-19). Written informed consent to use anonymously clinical data and samples was obtained from all eligible participants before inclusion.

All experiments were performed in accordance with relevant guidelines and regulations (Declaration of Helsinki).

Consent for publication

Not applicable

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

This study was funded by Occitanie region (Readynov R22064) and FONROGA foundation.

Authors' contributions

C.R.S., C.P., and F.G. designed and planned the study. T.M., C.P., and L.P.L. recruited patients for the study. R.D., D.S., and C.R.S. designed methodology. R.D. performed organoid culture. R.D., C.D. and C.R.S. performed data analysis and worked on figure generation. T.M. provided clinical data for the data analysis. R.D., D.S., and C.R.S. performed the literature review. C.P. and C.R.S. drafted the manuscript sections. C.D., N.V., F.G., T.M., C.P., C.R.S. edited and provided a critical review of the manuscript. All authors have read and approved the final version of the manuscript.

Acknowledgements

We thank the Centre de Ressources Biologiques (CRB) of ICM (Montpellier Regional Cancer Institute) for handling, freezing, data collection and shipment of patients' samples.

References

1- Edwards GO, Li Causi FS, Castagnola G, Singh S, Amendola PG. From mono- to multi-cellular *in vitro* models: reconstructing colorectal cancer complexity for translational and personalized applications. *Front Cell Dev Biol*. 2026;14:1777400. doi: 10.3389/fcell.2026.1777400.

2- Ni D, Xing J, Niu G, et al. Patient-derived organoids (PDOs): a novel preclinical platform to overcome challenges in cancer immunotherapy. *Front Cell Dev Biol*. 2026;14:1757516. doi: 10.3389/fcell.2026.1757516.

3- Tsai YH, Czerwinski M, Wu A, et al. A Method for Cryogenic Preservation of Human Biopsy Specimens and Subsequent Organoid Culture. *Cell Mol Gastroenterol Hepatol*. 2018;6(2):218-222.e7. doi: 10.1016/j.jcmgh.2018.04.008.

4- Chen P, Zhou JB, Chu XP, et al. Establishing a cryopreserved biobank of living tumor tissues for drug sensitivity testing. *Bioact Mater*. 2025;46:582-596. doi: 10.1016/j.bioactmat.2024.09.008.

5- Sébert M, Denadai-Souza A, Quaranta M, et al. Thrombin modifies growth, proliferation and apoptosis of human colon organoids: a protease-activated receptor 1- and protease-activated receptor 4-dependent mechanism. *Br J Pharmacol*. 2018;175(18):3656-3668. doi: 10.1111/bph.14430.

6- D'Aldebert E, Quaranta M, Sébert M, et al. Characterization of Human Colon Organoids From Inflammatory Bowel Disease Patients. *Front Cell Dev Biol*. 2020;8:363. doi: 10.3389/fcell.2020.00363.

7- Laborde N, Barusseaud A, Quaranta M, et al. Human colonic organoids for understanding early events of familial adenomatous polyposis pathogenesis. *J Pathol*. 2025;265(1):26-40. doi: 10.1002/path.6366.

8- Fujii M, Shimokawa M, Date S, et al. A Colorectal Tumor Organoid Library Demonstrates Progressive Loss of Niche Factor Requirements during Tumorigenesis. *Cell Stem Cell*. 2016;18(6):827-838. doi: 10.1016/j.stem.2016.04.003.

9- Dunne PD, Arends MJ. Molecular pathological classification of colorectal cancer-an update. *Virchows Arch*. 2024;484(2):273-285. doi: 10.1007/s00428-024-03746-3.

10- Marefati M, Fernandez-Vallone V, Leprovots M, et al. A Lgr5-independent developmental lineage is involved in mouse intestinal regeneration. *Development*. 2025;152(7):dev204654. doi: 10.1242/dev.204654.

11- Calafato G, Alquati C, Bernardi A, Di Paola FJ, Ricciardiello L. Comparative Analysis of Commercial and Home-Made Media on RSPO1/S6R Axis in Organoids with Different Wnt Backgrounds: A Methodological Guide for the Selection of Intestinal Patient-Derived Organoids Culture Media. *Int J Mol Sci*. 2024;25(21):11526. doi: 10.3390/ijms252111526.

12- Aran D, Camarda R, Odegaard J, et al. Comprehensive analysis of normal adjacent to tumor transcriptomes. *Nat Commun*. 2017;8(1):1077. doi: 10.1038/s41467-017-01027-z.

13- Cartry J, Bedja S, Boilève A, et al. Implementing patient derived organoids in functional precision medicine for patients with advanced colorectal cancer. *J Exp Clin Cancer Res*. 2023;42(1):281. doi: 10.1186/s13046-023-02853-4.

14- Lee MS, Menter DG, Kopetz S. Right versus left colon cancer biology: Integrating the consensus molecular subtypes. *J Natl Compr Canc Netw* 2017; 15(3): 411. doi: 10.6004/jnccn.2017.0038.

324