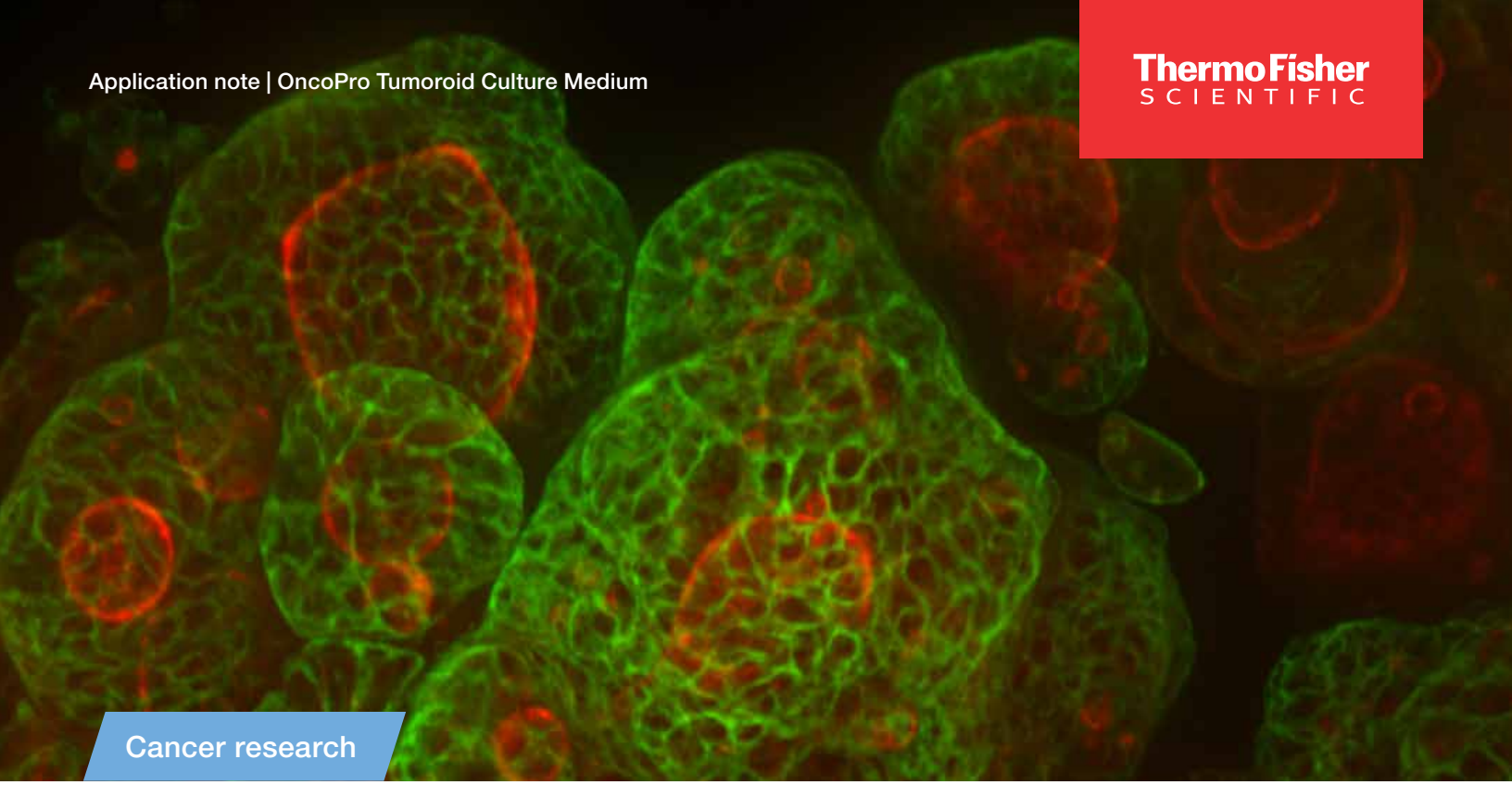

Cancer research

Tumoroid line derivation using OncoPro Tumoroid Culture Medium

Introduction

Tumoroids, also known as cancer organoids, are patient-derived cancer cells grown as 3D, self-organized multicellular structures. While commercial tumoroid lines are available, it may be desirable to derive novel tumoroid lines from patient tumor tissue. We classify a primary tumoroid culture as a tumoroid line if the culture:

- Maintains patient-specific characteristics
- Is subcultured at least once every 14 days for >5 passages
- Has achieved >5 cumulative population doublings since culture initiation
- Is freeze-thaw competent

Derivation success rates vary widely and multiple factors impact tumoroid line establishment, including cancer indication, time between collection of the tumor sample and culture initiation, method of tissue dissociation, size of the tumor sample, and number of cancer cells within the sample.

Although Gibco™ OncoPro™ Tumoroid Culture Medium was developed for the expansion of established tumoroid lines, we have used it to successfully derive new lines. Here we provide a summary of our methods and results in deriving new tumoroid lines with the OncoPro Tumoroid Culture Medium (Figure 1), as well as some lessons learned based on our experience.

Figure 1. Recommended workflow schematic for establishing tumoroid lines from tumor samples.

Recommended workflow

Source tumor sample

Tumoroid lines can be derived from patient tumor samples or patient-derived xenografts. Greater derivation success was achieved with fresh tissue than with viably cryopreserved tumor samples. The tumor tissue was stored on ice in a cold transport medium (e.g., Gibco™ Hibernate™-A Medium) immediately following resection. We found that minimizing the time between tumor resection and initiation of culture (ideally <24 hours) is a key to successful tumoroid line establishment.

Product highlight

Gibco™ Hibernate™-A Medium—This medium is independent of CO_2, enabling sensitive cells to be maintained in ambient CO_2 levels for up to 48 hours. This feature makes the medium an exceptional choice for transporting tumor tissue from the site of tumor collection to the cell culture laboratory for processing.

Dissociate tumor tissue

Upon arrival in the laboratory, the tumor tissue was minced into ~2 mm wide chunks with a scalpel (Figure 2), washed in Gibco™ Hank's Balanced Salt Solution supplemented with antibiotics, and dissociated using the appropriate enzymatic dissociation reagent for our tissue of interest. We found that it is important to optimize the type (e.g., collagenase, Gibco™ TrypLE™ Express Enzyme) and concentration of dissociation enzyme, as well as the exposure time for each cancer indication. We dissociated the tissue under agitation until we had a mixture of single cells and small clusters of a few cells. In this study, over-dissociation of the tissue (e.g., complete singularization) decreased the chance of a successful derivation.

Product highlight

Gibco™ Collagenase, Type IV, powder—The optimal type and concentration of dissociation reagent for tissue is dependent on the cancer indication. However, Collagenase Type IV may be a good starting place, as it is relatively gentle, to help prevent over-dissociation of the tissue and to release cell clusters (which could positively impact cell viability and tumoroid line establishment).

Initiate cell clusters

After dissociation, cells were separated from undigested tissue with a 100 µm cell strainer, washed further to remove dissociation enzymes, and then plated in complete OncoPro Tumoroid Culture Medium. Prior to plating, we recommend freezing a small portion of the cells (e.g., cell pellet and/or cells in Invitrogen™ TRIzol™ Reagent) for subsequent characterization by sequencing. We found that initial cell viability varies and is highly dependent on the initial tissue sample and dissociation conditions; additionally, initial cell viability did not seem to correlate strongly with derivation success. After dissociation, we maintained cells in suspension culture at 2.5 x 10^5 cells/mL (complete medium plus 2% basement membrane extract, BME) for 2–7 days to form small clusters.

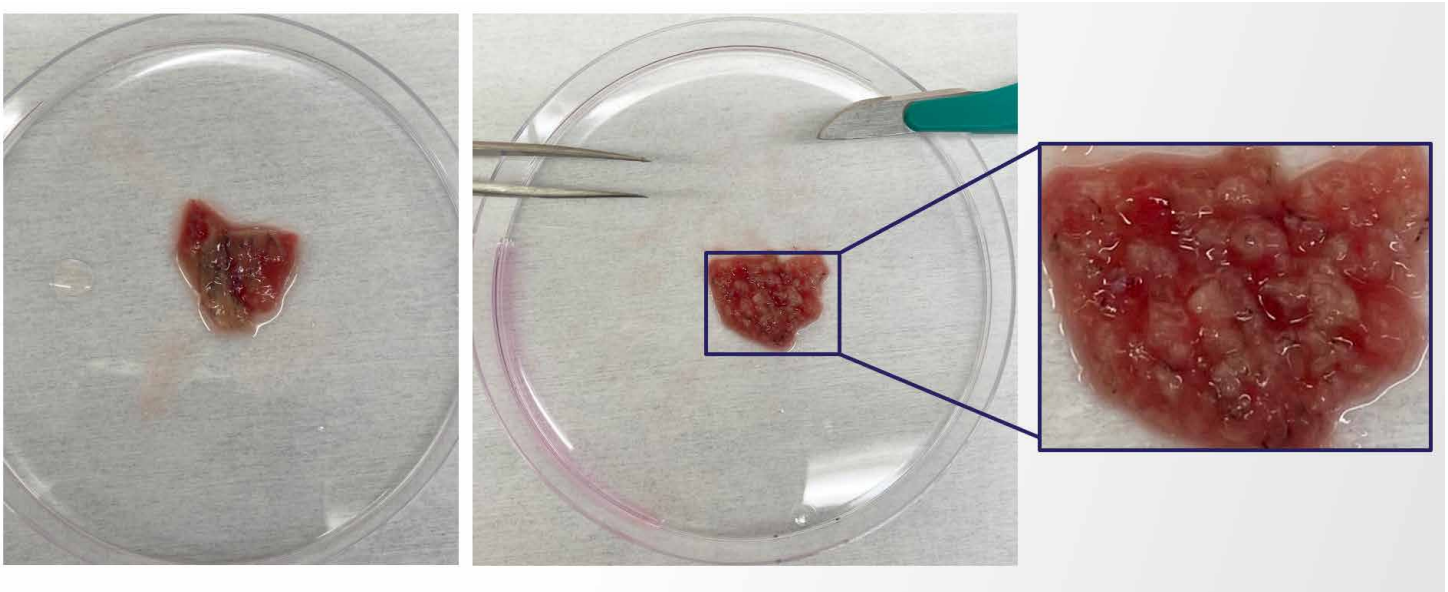

Figure 2. Tissue handling prior to dissociation. Tissue from a resection was received, washed, and minced before enzymatic dissociation.

Embed and expand

After small clusters and/or tumoroids formed, they were collected and replated in BME domes for initial expansion in embedded culture (typically 2–4 passages), until stable proliferation rates were obtained; additional details on embedded culture can be found in the **user guide**. In many cases, viable cell numbers decreased over the first several passages during derivation of new tumoroid lines. When derivation was successful, tumoroids then began expanding with a relatively stable doubling time, and could be transitioned to suspension culture for easier scale-up.

Scale up in suspension

We recommend suspension culture for scaling up cultures for bank establishment once consistent cell proliferation is achieved. Prior to transitioning to suspension culture, we recommend cryopreserving at least two vials of $\geq 2 \times 10^6$ viable cells from embedded cultures before plating $\geq 2.5 \times 10^5$ viable cells in the suspension format (2.6×10^4 viable cells/cm^2). Additional guidance for transitioning embedded cultures to suspension culture can be found in the **user guide**. We have found that tumoroids will consistently expand and maintain patient-specific characteristics in both suspension and embedded methods, though some cultures will grow more quickly in one method compared to the other. At the time of cryopreservation of the master bank, we recommend freezing additional samples of the cells for characterization by sequencing and comparison to the original (uncultured) dissociated tumor cells.

Additional tips

- When deriving multiple tumoroid lines concurrently, we recommend handling unique cultures separately. Additionally, we advise wiping down the shaft of all micropipettes with 70% ethanol or isopropanol before and after handling each tumoroid culture. This will lower the risk of cross-contamination of tumoroid cultures, especially as some cultures may be particularly slow-growing during early establishment.
- Antibiotics should be used to help reduce the risk of contamination of tumoroid cultures from the tissue sample.
- Tumoroid lines may be established in suspension culture, without embedding, for some cancer indications and/or patient samples. However, using embedded culture until consistent proliferation is observed over the course of several passages may be helpful in achieving higher derivation success rates.
- Tumoroid lines may be derived from viably cryopreserved dissociated tumor cells (DTCs) for some cancer indications and/or patient samples. If using DTCs as a source, we recommend thawing cells, quantifying viable cell number, and plating 2.5×10^5 cells/mL in suspension culture using complete medium plus 2% BME for 2–7 days to form small clusters prior to proceeding with embedded culture as described above. Tumoroid line derivation using fresh tissue as a source may be faster than starting with cryopreserved material.
- Samples should be characterized (e.g., mutational profile, short tandem repeats) upon culture initiation (by the collection of dissociated cells) and after establishing cryopreserved master banks. Additionally, when multiple tumoroid cultures are established concurrently, samples from master banks should be thawed, cultured for 3–4 passages, and compared to the bank to ensure that no contamination with another tumoroid line has occurred.

Product highlight

Ion Torrent™ Oncomine™ Comprehensive Assay v3—This targeted next-generation sequencing (NGS) assay enables the detection of relevant single-nucleotide variants (SNVs), copy number variations, gene fusions, and indels from 161 unique cancer-related genes. By comparing the results of this assay for the dissociated cells (collected prior to culture) and your cell bank, you can ensure that the mutational profile of the cells collected from the tumor matches that of your derived tumoroid line.

Results

We used OncoPro Tumoroid Culture Medium to derive tumoroid lines representing multiple cancer indications. Derivation success rates can be quite low and are highly dependent on several factors, including quality of the tissue, time from resection to culture, and cancer indication (Figure 3). Additionally, tumoroid line derivation can take anywhere from 6 to 12 weeks, and sometimes even longer (Figure 3). To get started with tumoroids more quickly, we recommend procuring established tumoroid lines.

Part of the characterization process during derivation is observing that patient-specific morphologies of tumoroids are maintained from initial culture through multiple passages (Figure 4). Tumoroids have a variety of morphologies, ranging from grape-like to solid to cystic. Some tumoroid cultures will exhibit innate heterogeneity of morphology within the same culture. We have shown that morphologies are maintained between our embedded and suspension culture methods.

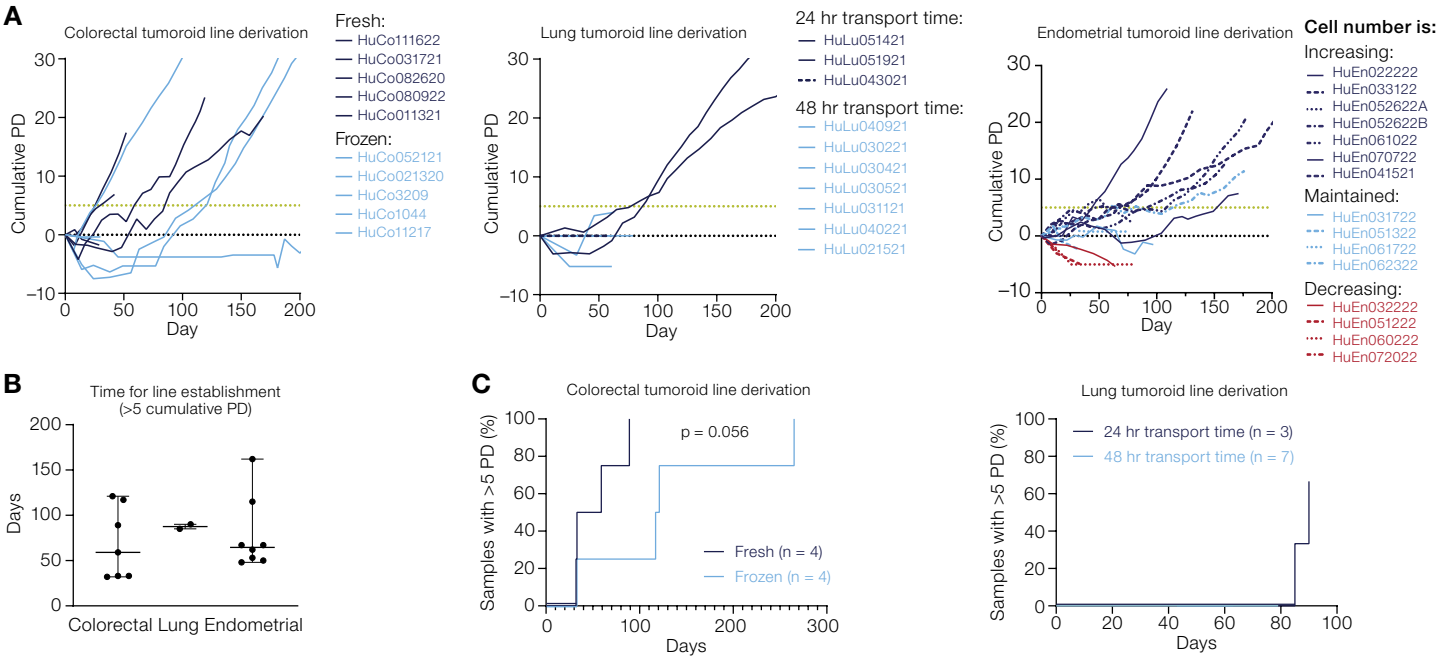

Figure 3. Tumoroid line derivation timeline and outcomes. (A) Cumulative population doublings (PD) over time during attempted derivation of colorectal, lung, and endometrial tumoroid lines. Factors that contributed to derivation success and time to establishment included whether the dissociated tumor cells had been cryopreserved prior to initiation of culture, time from resection to culture (e.g., extended shipping duration resulted in culture failure), and patient-to-patient variability (dotted black line: 0 cumulative PD, dotted green line: 5 cumulative PD). **(B)** Time for establishment of a tumoroid line is highly variable and can be upwards of 12 weeks in some cases. Error bars indicate the median ±95% confidence interval. **(C)** Comparison of time for successful derivation showed that beginning with fresh tissue can lead to faster tumoroid line establishment (left), though tumoroids can also be derived from viably cryopreserved dissociated tumor cells. Additionally, minimizing transport time between sample collection (e.g., tumor resection) and culture can increase the likelihood of culture establishment (right). Samples that did not re-form tumoroids after passaging or did not exhibit growth of individual tumoroid structures for >4 weeks were discarded.

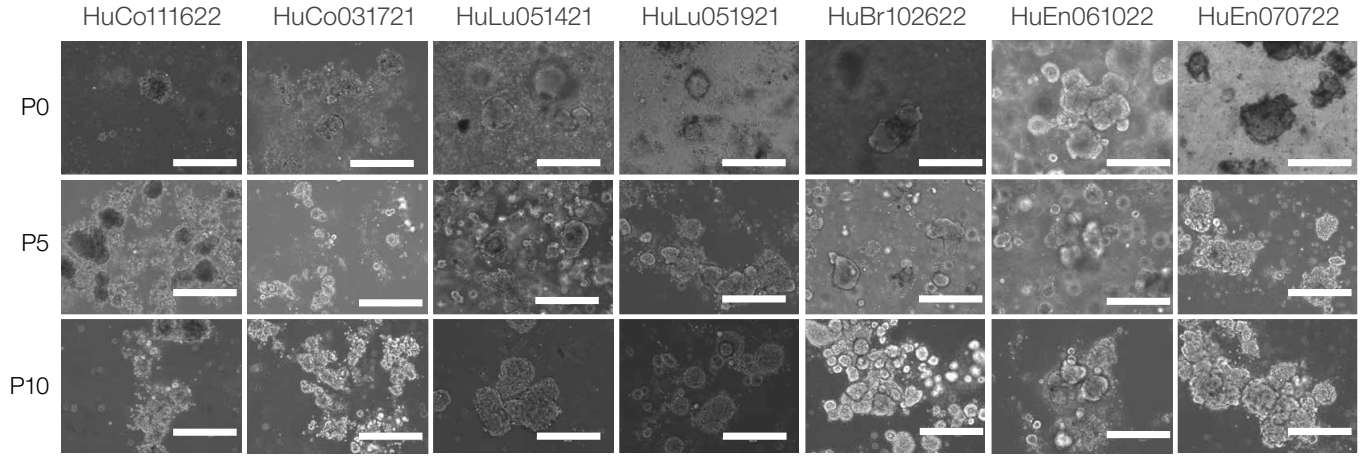

Figure 4. Tumoroids retained their morphology in culture. Representative images of tumoroid lines cultured in OncoPro Tumoroid Culture Medium at passage (P) 0, 5, and 10. Passage 0 represents only 1–2 weeks of culture. P0 and P5 images represent a mix of embedded and suspension samples. All P10 images are suspension cultures (HuCo = colorectal; HuLu = lung; HuBr = breast; HuEn = endometrial; scale bar = 400 µm).

A key benefit of tumoroid cultures is that they maintain patient-specific characteristics, including the germline and somatic mutations of the original tumor from which they were derived. We recommend that DNA from dissociated tumor cells collected prior to culture and post-bank establishment of the tumoroid line are sequenced and compared to ensure that the mutational profiles are conserved. This helps confirm the physiological relevance of the tumoroid line that has been established. We have observed high retention of SNV presence and allelic frequency during establishment of tumoroid lines in OncoPro Tumoroid Culture Medium across several cancer indications (Figure 5).

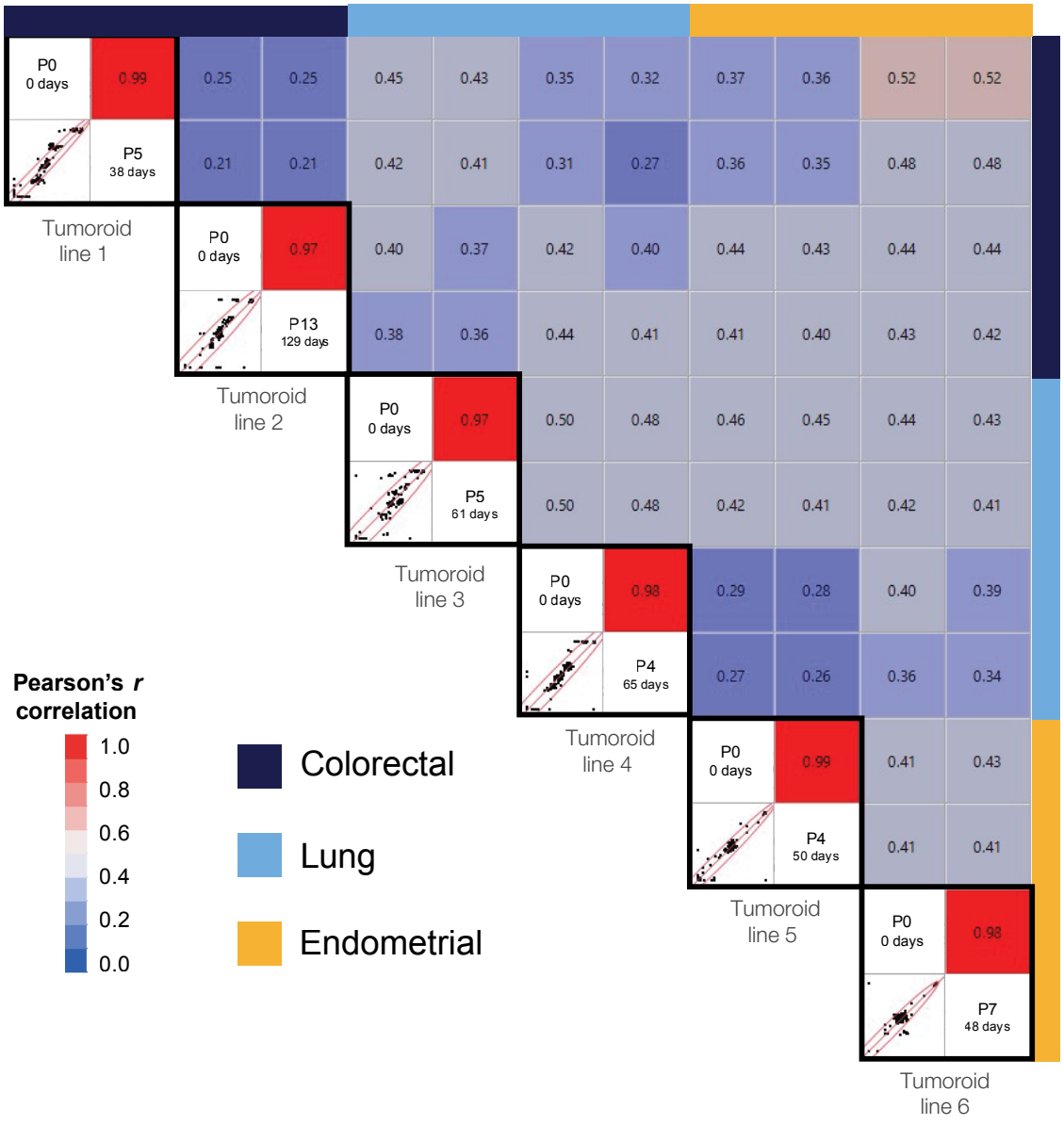

Figure 5. Tumoroid lines derived in OncoPro Tumoroid Culture Medium maintained patient-specific mutational profiles. Correlation matrix of SNVs between tumor tissue (P0) and tumoroid cultures after culturing long enough to cryopreserve more than five million cells. Each dot represents the variant allele frequency (VAF) for 1 genetic locus covered by the Oncomine Comprehensive Assay v3.

One occurrence during derivation that should be noted is the loss of the nonmalignant cell population as the tumoroid line is established. OncoPro Tumoroid Culture Medium was developed to selectively expand the malignant cell population from the original tumor (Figure 6). The loss of immune, endothelial, and other nonmalignant cell populations is evidenced by enrichment of the epithelial cell adhesion molecule (EpCAM)-positive population by flow cytometry, imaging, and differential gene expression analysis of cells collected from the tumoroid lines compared to the uncultured dissociated tumor cells from which they were established (Figure 6).

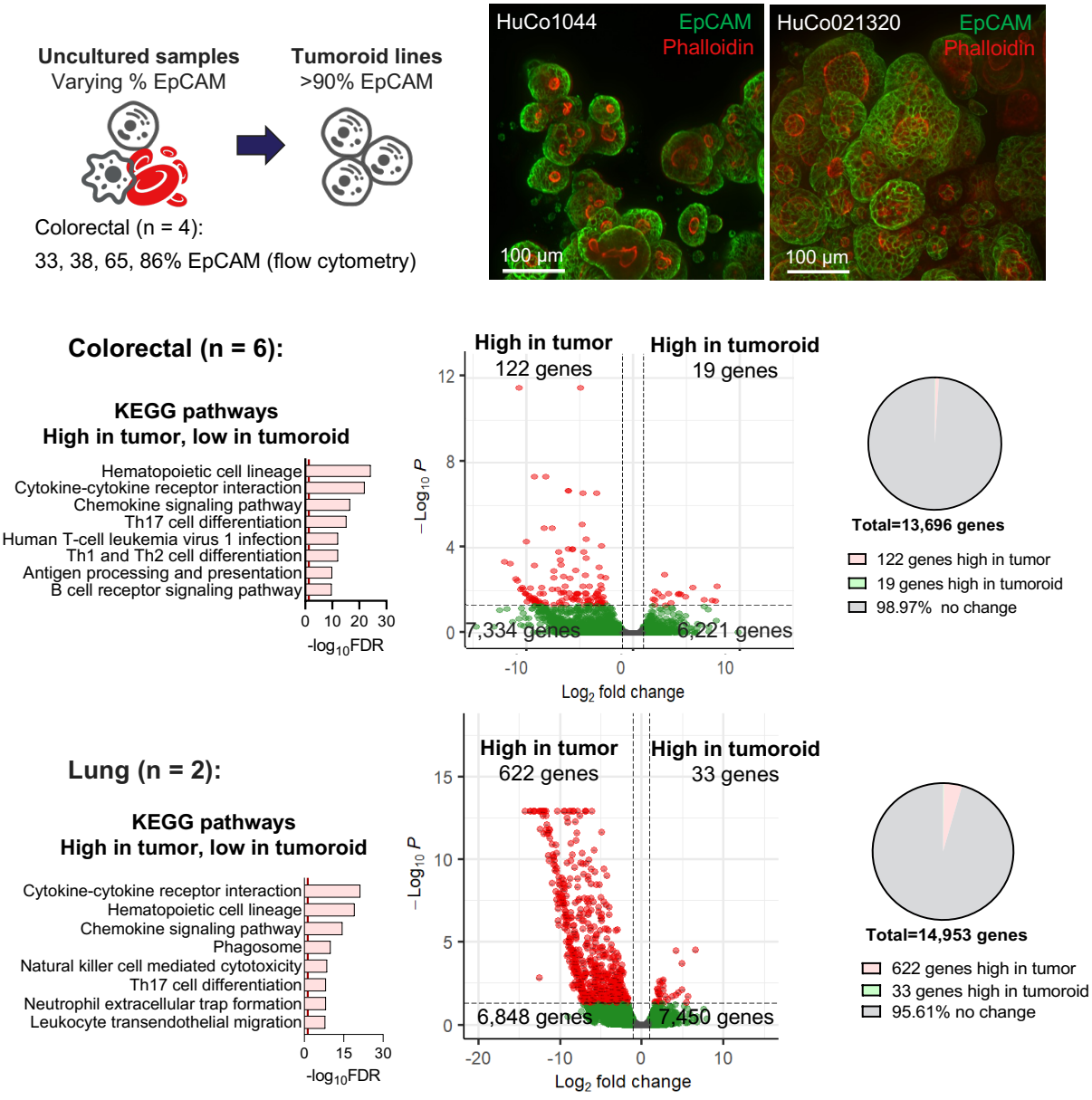

Figure 6. Transcriptomic comparison of primary tumors and early-passage tumoroids in colorectal and lung cancer indicates reduction in immune cell content. Tumor samples contain nonmalignant immune, stromal, and endothelial cells that are not supported long-term by the medium. Tumoroid lines are highly enriched for epithelial cell adhesion molecule (EpCAM)-positive tumor cells, as demonstrated by immunofluorescence and flow cytometry. Differential gene expression analysis revealed differentially expressed genes at a >2-fold change and a false discovery rate (FDR) of <0.05. Gene ontology revealed significantly enriched Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways at P0 (FDR <0.05), primarily relating to immune cell function. Bulk RNA sequencing was performed using the Ion AmpliSeq™ Transcriptome Human Gene Expression Panel, Chef-Ready Kit.

Conclusions

While OncoPro Tumoroid Culture Medium was initially developed for expanding existing tumoroid lines, the results presented in this application note demonstrate proof-of-concept compatibility with the derivation of new tumoroids from patient tissue. Researchers should be aware that derivation success rates can be variable and are influenced by factors such as tissue quality, time from tissue sample collection to culture, and the specific cancer indication. For the best chance of success, we recommend:

- Using fresh tissue that is processed <24 hours after sample collection
- Optimizing dissociation protocols for the tissue type of interest
- Plating dissociated tumor cells in suspension (plus BME) for 2–7 days to encourage cell cluster development
- Embedding in BME domes until stable growth rates are achieved
- Further expansion in suspension culture for greater ease-of-use

Despite the challenges associated with derivation, patient-derived tumoroid lines are valuable, physiologically relevant cancer models. Streamline your tumoroid culture workflow with the Gibco™ OncoPro™ Tumoroid Culture Medium Kit.

For more information on OncoPro Tumoroid Culture Medium please visit **thermofisher.com/oncopro**. For additional questions and support regarding tumoroid culture, please visit our tumoroid culture educational page at **thermofisher.com/tumoroid** to explore resources or **contact technical support**.

Ordering information

Product	Cat. No.
OncoPro Tumoroid Culture Medium Kit	A5701201
OncoPro Tumoroid Cell Lines	Submit an inquiry
Heat Stable FGF-10 Recombinant Protein	PHG0371
Geltrex LDEV-Free Reduced Growth Factor Basement Membrane Matrix	A1413201
StemPro Accutase Cell Dissociation Reagent	A1110501
TRIzol Reagent	15596026
Hibernate-A Medium	A1247501
Hanks' Balanced Salt Solution (no calcium, no magnesium)	14175095
Collagenase Type IV	17104019
TrypLE Express Enzyme	12604013
Nunc Non-treated Flasks	156800
Nunc Non-Treated Multidishes	150239
Nunc 15 mL and 50 mL Conical Sterile Polypropylene Centrifuge Tubes	339653
Oncomine Comprehensive Assay v3C	A35806
Ion AmpliSeq Transcriptome Human Gene Expression Panel, Chef-Ready Kit	A31446

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