Trevigen's Cultrex® Basement Membrane Extract (BME) for in vitro Organoid/Spheroid Studies

Hynda Kleinman, Ph.D., Jay George, Ph.D., and Gabriel Benton, Ph.D. Trevigen, Inc. Gaithersburg, MD

There is a growing recognition of the role of the extracellular matrix (ECM). The ECM is the biologically active macromolecular component present in all tissues and organs. It not only provides structural support for tissues and organs but contains information that controls cellular programs, differentiation, and morphogenesis. It is not surprising that different tissues and organs contain different ECMs. For example the ECM of the intestine is very different from that of the bone. Even the ECM within the same organ may be different. Evidence suggests that the ECM differs between intestinal crypts and intestinal villi. It is now understood that the mechanical properties of the matrix have a role in cellular and tissue dynamics. The tensile strength of the ECM, which is a function of its components, regulates cell adhesion, chemotaxis, migration, and orchestrated tissue and organ development [1].

Given that the ECM plays an important role in maintaining tissue and organ structure, it is no wonder that most 3D culture strategies rely on purified preparations of the ECM or

Figure 1: Time course of gastric organoid formation.

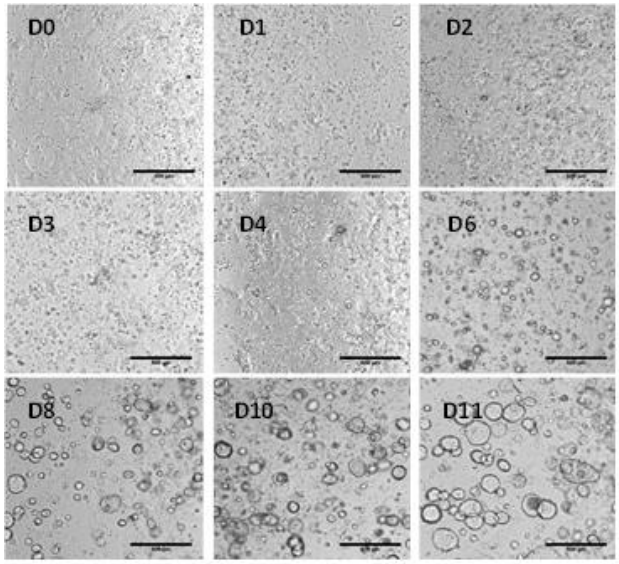
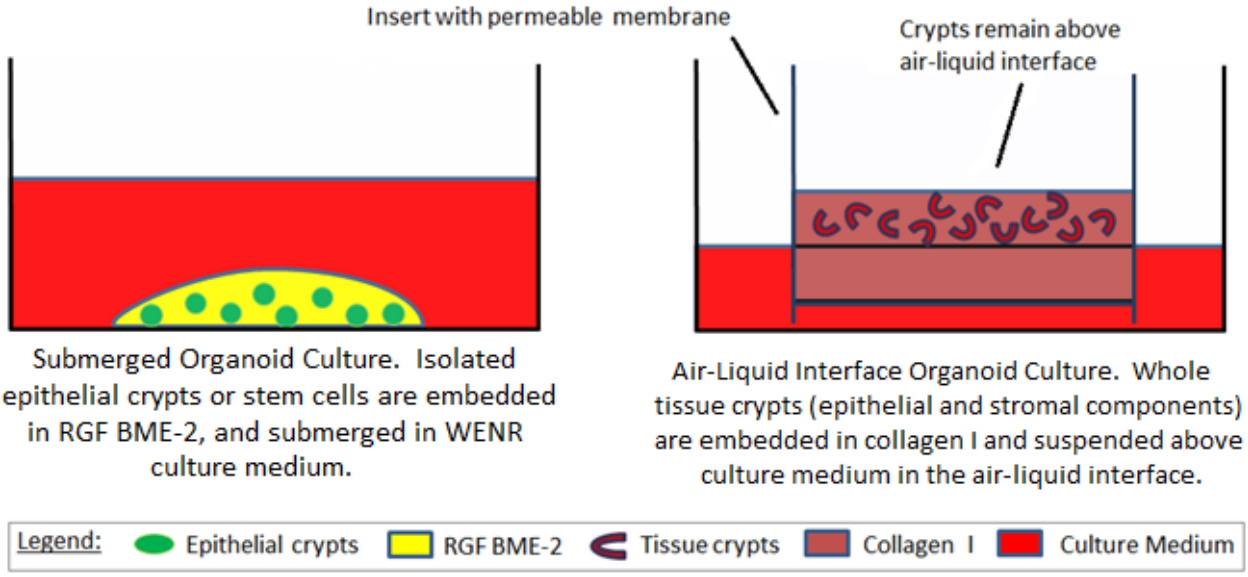
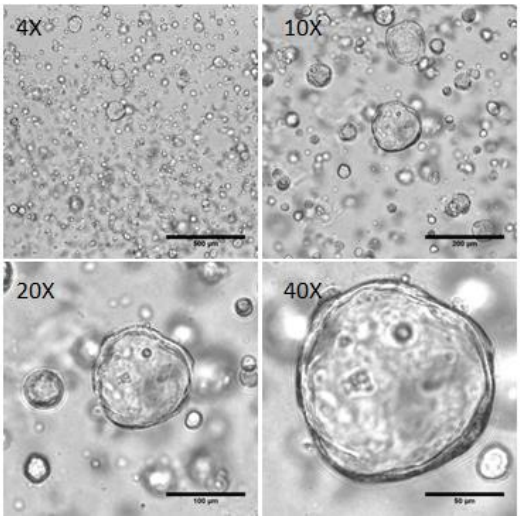


Figure 2: Schematics of standard organoid cultures in BME2 and at the air liquid interface (ALI).



In standard culture, the cells or tissue are place inside BME, plated on a dish where the BME gels, and then media is added. An organoid forms within days. For the air liquid interface culture, cells or tissue fragments are plated on top of gelled collagen I in an insert which is placed into a chamber with media such that the organoids are at the air liquid interface.

Figure 3: Gastric organoids formed at day 10 in 3D culture.

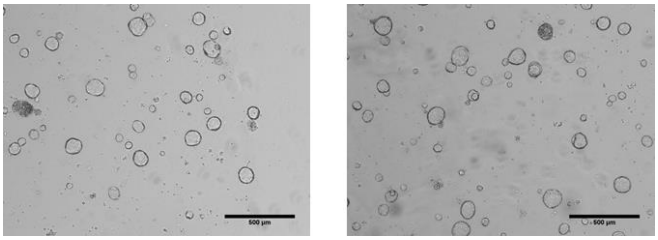


its individual components to provide an in vitro environment that more closely resembles physiologic conditions compared to traditional tissue culture approaches. The problem confronted by the research community is that companies that supply preparations of ECM to date have adopted a "one size fits all mentality". Scientists who purchase materials are forced to use the same basic ECM preparations regardless of the tissue or organ model under study. In recognition of the problem Trevigen is providing the research community with ECM preparations known as Trevigen's Cultrex® Basement Membrane Extract (BME) tuned for specific 3D culture applications.

Here we describe the use of BME for the generation of organoids* and spheroids** in 3D culture. Such in vitro organoid models of self-organized tissues and malignant tumors are useful for studying tissue development, tissue renewal, regenerative medicine, screening for drug toxicity, drug discovery, tumor therapeutic testing, etc [2-10].

Traditional 2D culture for studies on development and for drug screening has significant shortcomings with regard to cell differentiation and for translation to the clinic. Organoids are simple, 3D in vitro systems composed of cells or tissues that self-form a physiological-like structure with complexity and heterogeneity similar to that of intact tissue over time (Figure 1) [11, 12]. Organoid-forming cells can be derived directly from patient tissues and are maintained and expanded in 3D culture [13-15]. In addition, stem cells (alone or in aggregates) as well as circulating cancer cells have been shown to generate organoids representative of their respective tissue or tumor type [16, 17]. The two predominant methods for organoid culture are submerged and air-liquid interface (ALI) (Figure 2) [2]. For submerged organoid cultures, epithelial stem cells are embedded in Cultrex® reduced growth factor basement membrane extract type 2 (RGF BME2) that is stiffer than regular BME and cultured in medium containing Wnt, epidermal growth factor (EGF), Noggin, and R-spondin 1 (Figure 3). It should also be noted that there is consistent behavior between the various lots of BME2 for organoid formation (Figure 4).

Figure 4: Appearance of organoids on two different batches of BME2.



Lot # 32877D15 Lot # 32983D15

Table.1 Examples of potential and current uses of organoids

For cancer organoids

- Drug testing of therapeutics, oncogenic factors, genes, etc. (can create banks of organoids for large scale testing
- Determine tumor heterogeneity: malignant activity of specific cell types, therapeutic responses of specific cell types, etc.

For organoids from normal cells/stem cells:

- Models of tissue development
- Toxicity testing
- Define genes important in specific developmental stages: overexpression, siRNA

For ALI, crypt tissue fragments containing both epithelial and stromal components are cultured within a collagen I hydrogel that is suspended above the culture medium level using a cell culture insert (Figure 2) [11]. Mixtures of cells provide specificity and a more physiologically relevant in vitro model. Various types of organoids that have been generated in 3D culture include thyroid, intestine, brain, liver, breast, pancreas, salivary gland, kidney, lung, etc.

The use of Trevigen's basement membrane extract (BME) for 3D organoid culture has enabled the formation of many different types of mini-organs and tumor types. Such in vitro structures have a variety of uses (Table 1) [4-10]. Tumor-derived organoids can be used to test potential therapeutic drugs, oncongenic factors, and gene function. Gene function can be tested by transfection for either overexpression or silencing. One group has been making organoid banks for large scale drug testing [18]. Tumor organoids can also be used to define tumor heterogeneity and to determine the responsiveness of different cell populations within the tumor. Organoids from normal tissues can be used to study tissue development and to define genes important in different stages of development [2, 19, 20]. Toxicity testing can also be done with tissue organoids [21]. Thus, organoids have many uses.

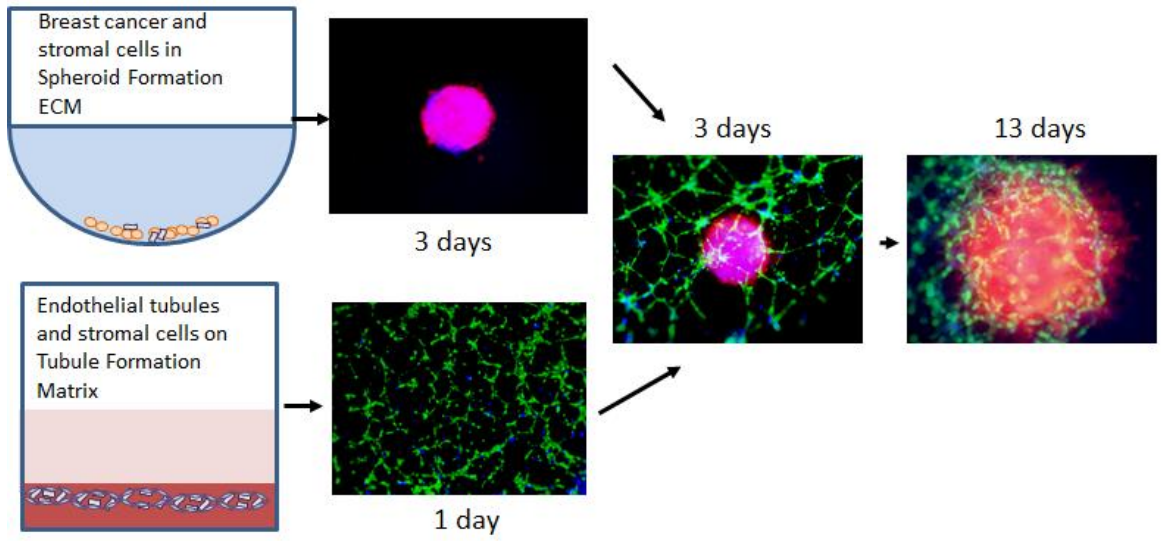
BME is composed of embryonic basement membrane components, including laminin I, collagen IV, entactin, heparan sulfate proteoglycan, and various growth factors [22]. BME provides both physical and chemical cues that facilitate

Table 2. Cultrex® Matrix and BME Products

Product*	Matrix, use
Cultrex® BME	Original, 2 and 3D culture
Cultrex® Reduced Growth Factor BME	Original, 2 and 3D culture
Cultrex® Stem Cell Qualified BME	2D stem cell culture
Cultrex® 3D Culture Matrix BME	3D culture
Cultrex® Organoid Qualified BME Type 2	Stiffer matrix, 3D organoid culture
Cultrex® Reduced Growth Factor Organoid BME Type 2	Stiffer matrix, 3D organoid culture
Cultrex® BME Type 3	Stiffer matrix, low pH, low glucose, tumor organoids and cells
Cultrex® 3D Culture Matrix Rat Tail Collagen I	Collagen I, ALI culture, tumor invasion when mixed with BME

*All are available in 1 and 5 ml aliquots | Protein concentration varies from 14-16 mg/ml | Tested and free of 31 pathogens (Pathclear®)

Figure 5: Steps for breast tumor organoid formation in triculture in a physiological tumor-aligned environment.



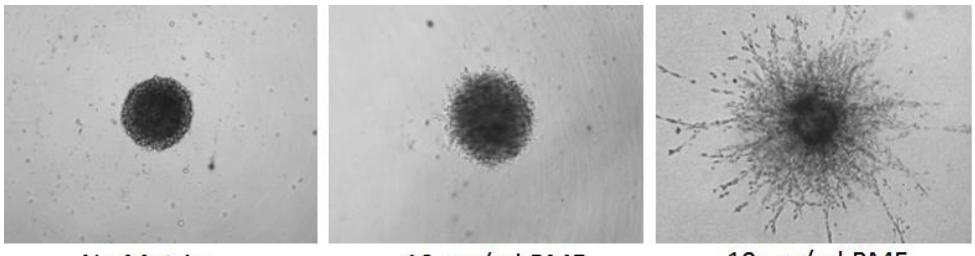
Breast tumor cells and stromal cells are mixed in a non adherent well with BME3 and allowed to form mixed organoids for up to 3 days. In parallel, endothelial cells and stromal cells are plated on gelled BME2 and allowed to form capillary-like structures for 12-24 hours. Then, the tumor/stromal cells organoids are transferred to the monolayer of capillary-like structures with stromal cells and cultured for additional time.

organoid formation. Cultrex® BME from Trevigen is available in several different compositions, including regular BME, BME type 2 (BME2) which is stiffer than regular BME, BME type 3 (BME3) which is stiffer, low pH, and low glucose, and many of these BMEs can be obtained with reduced levels of growth factors (Table 2). These various compositions are available to provide a physiologically relevant environment to promote organoid assembly and tissue-specific behavior.

Organoid formation of tumor cells and tissues has been used in studying tumor growth, invasion, drug sensitivity, and gene expression and is the driving force in future precision based medical approaches. Such an in vitro 3D model system is advantageous over in vivo systems in terms of cost, time, ability to screen large numbers of therapeutics, and potentially more reliable translation to the clinic. Currently, the in vivo screening required by regulatory agencies has proven to show limited success in the clinic with less than 1 drug in 10 obtaining regulatory approval. An important requirement of tumor organoids for drug testing includes an appropriate physiological microenvironment of cells, extracellular matrix factors, matrix stiffness, nutrient gradient, low pH, low oxygen, etc. A big advantage is that these and many other parameters can be manipulated in the 3D culture models to obtain a physiologically relevant and potentially personalized assay system for diagnostic and therapeutic testing. An exciting new tumor organoid system involving self-organized breast cancer cell spheroids in Cultrex®3D BME3 (tumor aligned matrix) co-cultured with stromal cells and endothelial cell tubes at low pH, low glucose, and low oxygen has been developed (Figure 5) [23]. In this model, the tumor cells together with mesenchymal stromal cells are mixed and allowed to self-organize in 3D BME culture. Then, the organoids are plated on a layer of already formed capillary-like structures of endothelial cells in 3D culture on BME. This triculture tumor system more closely mimics the in vivo tumor microenvironment and has demonstrated significant differences in drug sensitivity and gene expression from that seen in 3D monoculture and in 2D culture. This system has the potential to be adapted to other tumor types, including cells isolated from patient tumors or to circulating tumor cells for personalized medicine. Clearly, the improved tissue architecture, tumor aligned microenvironment, and cellular organization of a growing and invading tumor mini-tissue provides a better therapeutic testing platform than that currently available. This system can also be scaled up for high-throughput screening.

Invasion can also be observed and measured with tumor organoids in 3D BME3 culture [23]. In the case of the breast cancer cells, the BME3 alone does not support invasion. Increasing amounts of collagen added to the BME3 will increase invasion (Figure 6) that can be easily measured. This system also allows for drug and gene testing to define inhibitors and stimulators of invasion.

Figure 6: Breast cancer cell invasion into collagen I-containing matrix.



Breast cancer cells exhibit differential invasion based on their microenvironment. MDA-MB-231 (3,000 cells/well) formed spheroids in low adhesion plates containing spheroid formation ECM for 72 hours. Then spheroids were embedded in A) No matrix, B) 10 mg/ml BME, or C) 10 mg/ml BME and 1 mg/ml collagen I. After hydrogel polymerization, DMEM, 10% FBS was added to each well to promote a chemotactic response over a 96 hour period. Note that on BME containing collagen I, the cells begin to invade the matrix.

Using Cultrex®BME2 in 3D culture, the Clevers group (Hubrecht Institute, Netherland) has been able to generate both normal- and tumor-derived intestinal organoids from both healthy and tumor tissue simultaneously from biopsy material from previously untreated colorectal cancer patients with 90% success [18]. In some cases, they were able to obtain thousands of organoids per biopsy, but some tissues only provided 10-20 primary organoids. The organoids from the normal tissue and from the tumor biopsies closely recapitulated the morphology of the normal tissue and of the parental tumor, respectively. Since tumors are heterogenous, there was a range of patient-specific morphologies. Furthermore, DNA from the primary tumor and the tumor-derived organoids were analyzed and found to have molecular signatures that in many cases correlated with drug sensitivities in a high-throughput screen of 83 compounds.

This small study with 18 patient samples and 83 tested drugs demonstrates the feasibility of using organoids in 3D BME culture for defining the molecular basis of drug responses. Clearly, a larger study is needed for this approach to be able to be used in personalized medicine. The most common methods for personalized medicine utilize mice bearing the patient's tumor for drug studies or tissue slices in culture. The former method suffers from the long time required to grow tumors in mice for drug testing while the slice culture method is problematic due to limited growth of these slices in vitro. The 3D BME organoid culture of patient samples is feasible, can define clinically relevant biomarkers, can determine drug sensitivities, and is adaptable to high-throughput screening. This model system may facilitate the identification of personalized therapeutics. These organoids with the relevant information on their molecular signatures and drug sensitivities could be stored as part of an organoid biobank.

In summary, we have presented important validation of the use of BME for organoid formation for studying tissue development and tumor modeling. These organoids reflect the tissue architecture and behavior of the parental tissue or tumor. Importantly, we believe that these new model systems will have value in therapeutic drug testing in cancer. Furthermore, the process of oncogenic transformation can also be studied in organoid culture and used for drug screening [13, 24].

*An organoid is a miniature organ in vitro derived from primary tissue stem cells or inducible pluripotent stem cells; organoid cultures mimic organ structure and function.
**A spheroid is a cluster of cells derived from cell lines that organize into a spherical aggregate.

References

1. Rozario, T. and D.W. DeSimone, *The extracellular matrix in development and morphogenesis: A dynamic view.* Developmental Biology, 2010. **341**(1): p. 126-140.
2. Ader, M. and E.M. Tanaka, *Modeling human development in 3D culture.* Curr Opin Cell Biol, 2014. **31**: p. 23-8.
3. Calderon-Gierszal, E.L. and G.S. Prins, *Directed Differentiation of Human Embryonic Stem Cells into Prostate Organoids <italic>In Vitro</italic> and its Perturbation by Low-Dose Bisphenol A Exposure.* PLoS ONE, 2015. **10**(7): p. e0133238.
4. Matano, M., et al., *Modeling colorectal cancer using CRISPR-Cas9-mediated engineering of human intestinal organoids.* Nat Med, 2015. **advance online publication**.
5. Sachs, N. and H. Clevers, *Organoid cultures for the analysis of cancer phenotypes.* Current Opinion in Genetics & Development, 2014. **24**: p. 68-73.
6. Skardal, A., et al., *Liver-Tumor Hybrid Organoids for Modeling Tumor Growth and Drug Response In Vitro.* Annals of Biomedical Engineering, 2015. **43**(10): p. 2361-2373.
7. Ranga, A., N. Gjorevski, and M.P. Lutolf, *Drug discovery through stem cell-based organoid models.* Advanced Drug Delivery Reviews, 2014. **69–70**: p. 19-28.
8. Vela, I. and Y. Chen, *Prostate cancer organoids: a potential new tool for testing drug sensitivity.* Expert Review of Anticancer Therapy, 2015. **15**(3): p. 261-263.
9. Cantrell, M.A. and C.J. Kuo, *Organoid modeling for cancer precision medicine.* Genome Medicine, 2015. **7**(1): p. 32.
10. Boj, Sylvia F., et al., *Organoid Models of Human and Mouse Ductal Pancreatic Cancer.* Cell, 2015. **160**(1â€“2): p. 324-338.
11. Sato, T. and H. Clevers, *SnapShot: Growing Organoids from Stem Cells.* Cell, 2015. **161**(7): p. 1700-1700.e1.
12. Sasai, Y., *Next-Generation Regenerative Medicine: Organogenesis from Stem Cells in 3D Culture.* Cell Stem Cell, 2013. **12**(5): p. 520-530.
13. Salahudeen, A.A. and C.J. Kuo, *Toward recreating colon cancer in human organoids.* Nat Med, 2015. **21**(3): p. 215-216.
14. Huch, M., et al., *Long-Term Culture of Genome-Stable Bipotent Stem Cells from Adult Human Liver.* Cell, 2015. **160**(1â€“2): p. 299-312.
15. Xia, Y., et al., *The generation of kidney organoids by differentiation of human pluripotent cells to ureteric bud progenitor–like cells.* Nat. Protocols, 2014. **9**(11): p. 2693-2704.

16. Gao, D., et al., *Organoid Cultures Derived from Patients with Advanced Prostate Cancer.* Cell, 2014. **159**(1): p. 176-187.
17. Fernandez-Perianez, R., et al., *Basement Membrane-Rich Organoids with Functional Human Blood Vessels Are Permissive Niches for Human Breast Cancer Metastasis.* PLoS ONE, 2013. **8**(8): p. e72957.
18. van de Wetering, M., et al., *Prospective Derivation of a Living Organoid Biobank of Colorectal Cancer Patients.* Cell, 2015. **161**(4): p. 933-945.
19. Ren, W., et al., *Single Lgr5- or Lgr6-expressing taste stem/progenitor cells generate taste bud cells ex vivo.* Proceedings of the National Academy of Sciences, 2014. **111**(46): p. 16401-16406.
20. Greggio, C., et al., *Artificial three-dimensional niches deconstruct pancreas development in vitro.* Development, 2013. **140**(21): p. 4452-4462.
21. Astashkina, A. and D.W. Grainger, *Critical analysis of 3-D organoid in vitro cell culture models for high-throughput drug candidate toxicity assessments.* Advanced Drug Delivery Reviews, 2014. **69–70**: p. 1-18.
22. Benton, G., et al., *Matrigel: From discovery and ECM mimicry to assays and models for cancer research.* Adv Drug Deliv Rev, 2014.
23. Benton, G., et al., *In Vitro Microtumors Provide a Physiologically Predictive Tool for Breast Cancer Therapeutic Screening.* PLoS ONE, 2015. **10**(4): p. e0123312.
24. Li, X., et al., *Oncogenic transformation of diverse gastrointestinal tissues in primary organoid culture.* Nat Med, 2014. **20**(7): p. 769-777.