3D culture models of normal and malignant breast epithelial cells

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ABSTRACT

Extracellular matrix is a key regulator of normal homeostasis and tissue phenotype[1]. These signals are lost when cells are cultured *ex vivo* on two dimensional (2D) plastic substrata. Many of these crucial microenvironmental cues may be restored using three dimensional (3D) cultures of laminin-rich extracellular matrix (lrECM)[2]. 3D culture assays enable phenotypic discrimination between non-malignant and malignant mammary cells, as the former grown in a 3D context form polarized, growth-arrested acinus-like colonies while the latter form disorganized, proliferative and non-polar colonies[3]. Signaling pathways which function in parallel in cells cultured on plastic become reciprocally integrated when the cells are exposed to basement membrane-like gels[4-7]. Appropriate 3D culture thus provides a more physiologically relevant approach to the analysis of gene function and cell phenotype *ex vivo*. We describe here a robust and generalized method for the culturing of various human breast cell lines in 3D and describe the preparation of cellular extracts from these cultures for molecular analyses.

MATERIALS

REAGENTS

- Engelbreth-Holm-Swarm extracellular matrix extract (EHS), growth factor-reduced (Matrigel™, BD Biosciences; Cultrex® BME, Trevigen)
- Hard-set mounting medium (VECTASHIELD HardSet, Vector Laboratories; ProLong Gold, Invitrogen)
- Immunofluorescence (IF) buffer: 0.2% Triton-X 100, 0.1% BSA (RIA grade), 0.05% Tween 20 in PBS (pH 7.4; sterilized, 0.22 μm filter); if long term storage is desired, add 7.7 mM NaN_3
- IF blocking solution: 10% goat serum, 1% goat $F(ab')_2$ anti-mouse IgG (Caltag) in IF buffer
- Phosphate-buffered saline (PBS): 130 mM NaCl, 13 mM Na_2HPO_4, 3.5 mM NaH_2PO_4
- PBS/EDTA: 5 mM EDTA, 1 mM $NaVO_4$, 1.5 mM NaF in PBS
- PBS/glycine: 100 mM glycine in PBS

PROCEDURE

Margin heading: Culturing cells in 3D

We utilize the term "3D culture" to refer to the culture of cells in the presence of a laminin-rich extracellular matrix (lrECM), in particular, the solubilized extract derived from the Engelbreth-Holm-Swarm mouse sarcoma (EHS)[8]. For a discussion of user options regarding 3D matrices, see ***Box 1****. The procedure below describes the 3D "embedded" assay, in which cells are cultured*

embedded in an lrECM gel[9] ***(Fig. 1)****. Alternatively, the 3D "on-top" assay, in which cells are cultured on top of a thin lrECM gel overlaid with a dilute solution of lrECM, may be used as described in* ***Box 2*** *(****Figs. 1*** *and* ***2****).*

1. Thaw EHS at 4°C overnight.

2. Coat pre-chilled culture surface (e.g. dish or well) with a thin layer of EHS. Slowly pipette the appropriate volume (**Table 1**) directly onto surface and spread evenly with a pipette tip or plunger of a 1 ml syringe for smaller areas or cell lifter for larger areas. Incubate for 15 to 30 min at 37°C to allow the EHS to gel (but do not let it overdry).

CRITICAL STEP

3. Trypsinize cells from a monolayer to a single cell suspension.

Use cells that are healthy and not more than 75% confluent.

TROUBLESHOOTING

4. Aliquot cells to be plated into a 1.5 ml microcentrifuge tube. Gently pellet the cells by centrifugation at ~115g and, on ice, resuspend the cells into the appropriate volume of EHS (**Table 1**).

The number of cells to be plated per ml of EHS will depend on the growth properties of the cell line and may need some optimization, but we recommend the following ranges: for non-malignant cells, 0.85 x 10^6 *cells/ml; for malignant cells, 0.50 - 0.60 x* 10^6 *cells/ml.*

CRITICAL STEP

5. Pipette the mixture of cells and EHS onto the pre-coated surface. Incubate 30 min at 37°C to allow EHS to gel. Add appropriate volume of cell line-appropriate medium (**Table 1**).

TROUBLESHOOTING

6. Maintain culture for 10 days, changing medium every 2 days.

7. To perform drug response assays in 3D, see **Box 3**.

Margin heading: Extracting cells from 3D cultures

8. Colonies may be fully extracted from their 3D culture environment for further analyses, e.g. immunostaining, DNA, RNA and protein extraction (**Option A**, Complete extraction). If only an immunostaining endpoint is desired, a complete extraction is often not necessary. An abbreviated procedure may be performed in which the gel is partially broken down, allowing for sufficient dissolution of polymerized EHS in the sample for immunostaining (**Option B**, In-well

extraction). Alternatively, colonies may be fixed and then immunostained directly in the gel (**Option C**, Whole culture fixation; proceeding to **Box 4**, Whole culture immunostaining). This latter method is generally only preferable if a 3D colony you would like to visualize would be disrupted by extraction (as in **Fig. 2c**) as the immunostaining procedure then requires a much larger amount of antibody per sample and is less flexible since a single culture may only be stained against one set of markers. If only RNA extraction is desired, the culture may be directly solubilized by addition of Trizol.

Option A Complete extraction

Perform all steps on ice.

a. Aspirate media, rinse 2X with 1 [media] volume ice-cold PBS.
b. Add 2-3 volumes cold PBS/EDTA. Detach EHS from the bottom of culture surface using a cell lifter (for dishes of diameter ≥ 35 mm) or by gently scraping the bottom with a pipette tip. Shake gently for 15-30 min.
   3D embedded cultures will require a larger volume of PBS/EDTA than 3D on-top cultures and will also take longer to break down.
c. Transfer solution to a conical tube. Rinse culture surface once with 0.5 volume PBS/EDTA, transferring the rinse also to the tube. Gently shake tube on ice for 15-30 min.
d. Inspect tube to check that EHS has dissolved completely (invert tube gently and look for a homogeneous suspension of cell colonies without visible EHS gel fragments).
   If not, wait longer and/or add additional PBS/EDTA.
e. To collect colonies for immunostaining, follow these steps. Otherwise, skip ahead to (f).
   i. Centrifuge at ~115g for 1-2 minutes such that cell colonies collect at the bottom of the tube but do not form a tight pellet.
      CRITICAL STEP
   ii. Carefully aspirate the majority of the supernatant. Gently resuspend the colonies. Pipette approximately 15 µl of the colony suspension

onto a slide and fix, using an fixative appropriate for the antigen of interest.

CRITICAL STEP

PAUSE POINT Colonies fixed on slides may be stored at -20°C for several months.

f. Centrifuge the colonies into a pellet. Aspirate supernatant and lyse with appropriate extraction buffer and process using standard procedures[10].
For protein extraction an additional wash with PBS/EDTA will minimize the amount of EHS in the final extract.
PAUSE POINT Extracts may be stored at -80°C for several months.

Option B In-well extraction

Perform all steps on ice.

a. Follow **Option A** [a-b]
b. Check under microscope to verify that majority of EHS has broken apart and colonies have settled to bottom of the well
If not, wait longer and/or add an additional volume PBS/EDTA.
c. Carefully aspirate the majority of the supernatant. Pipette approximately 15µl of the colonies in solution onto a slide and fix.
CRITICAL STEP
PAUSE POINT Cultures fixed on slides may be stored at -20°C for several months.

Option C Whole culture fixation

Perform all steps on ice.

a. Aspirate media, rinse 2X with 1 [media] volume ice-cold PBS.
b. Fix culture with 4% paraformaldehyde at room temperature for 10 minutes.
c. Stop fixation with PBS/glycine for 10 minutes; wash once and store in PBS.
PAUSE POINT Fixed cultures may be stored at 4°C for up to 4 days.

Margin heading: Immunostaining of 3D cultures

9. This immunostaining procedure applies to cultures fixed on slides generated by **Options A** or **B** (**Figs. 2a**, **2b** and **3**) but may also be applied to the immunostaining of fixed whole cultures generated by **Option C** (see **Box 4**, Whole culture immunostaining; **Fig. 2c**).

10. Wash slides: PBS/glycine, 3X 10 min, room temperature (RT).

11. Block slides: IF blocking solution, 1.5h, RT in moist chamber.

The anti-mouse F(ab')$_2$ fragments in the IF blocking solution block against immunoreactive mouse IgG species in EHS. 100 µl of blocking solution spread with a strip of Parafilm is sufficient volume for incubation.

12. Primary antibody staining: Primary antibody of interest diluted in IF buffer, 2h, RT in humid chamber.

13. Wash slides: IF buffer, 3X 20 min, RT.

14. Secondary antibody staining: Secondary antibody diluted in IF buffer, 45 min, RT in humid chamber.

15. Wash slides: IF buffer, 1X 20 min; PBS 2X 10 min, RT.

16. Nuclear counterstain: DAPI (0.5 µg/ml), 5 min, RT.

17. Wash slides: PBS, 10 min, RT.

18. Mount slides: Spread one drop of hard-set mounting medium with a No. 1 ½ coverslip and allow to set overnight at 4°C.

Take care to avoid forming bubbles when mounting to minimize photobleaching.

PAUSE POINT Mounted slides may be stored at -20°C for several months.

19. Due to the thickness of the samples, confocal microscopy is ideal for imaging of 3D cultures.

TROUBLESHOOTING TABLE

PROBLEM	SOLUTION
Step 5 Cells are not suspended and have settled to the bottom of the EHS gel	If the cell/EHS mixture in **Step 4** is diluted with medium prior to setting, the cells may settle to the bottom of the gel. Be sure to aspirate the majority of the supernatant to avoid this problem.

Step 19 Excess EHS causes immunostaining background.	Colonies fully extracted from the EHS gel as in **Option A** should have little or no background. Partial extraction of colonies by **Option B** may result in background if EHS is not sufficiently broken down, and a haze or cloud of EHS adheres to cells. To avoid this, increase both the volume of PBS/EDTA used and the incubation time.
Box 2 Step 4 After plating, cells are not resting singly on the layer of EHS but have aggregated together in clumps and/or are concentrated in the center of the well.	Cells of some lines tend to aggregate with one another and may not adhere as quickly to the EHS. In **Step 4**, agitation of the plate in the xy plane at intervals during incubation at 37°C may assist with preventing cell concentration in the center of the well (do not apply a swirling motion as cells will then accumulate around the edge of the well).

CRITICAL STEPS

Step 2 Culture surfaces must be pre-chilled and coated on ice to ensure even spreading of EHS. Pipette EHS slowly and directly onto culture surface to avoid formation of bubbles which may allow cells to come in direct contact with the culture surface and begin to spread as a monolayer beneath the gel.

Step 4 After aspiration of the supernatant, gently flick the tube to loosen the cell pellet so that when EHS is added, the cells are in a single cell suspension. Pipette carefully when mixing to avoid bubbles.

Step 8, Option A (e, i) Centrifugation time will depend on the size of your colonies and may require some optimization. Larger colonies will settle on their own and may only require a pulse to collect at the bottom of the tube whereas smaller colonies may have only just collected in the conical area of the tube after 2 minutes of centrifugation.

Step 8, Option A (e, ii); Option B (c) The amount of aspiration required to achieve the balance between getting a high number of colonies on the slide in a relatively low volume of liquid may

require some practice. If the volume of liquid used to pipette a sufficient number of colonies is too high it may decrease the efficiency of fixation. The slides may sit for some time to allow excess liquid to evaporate but do not allow the cells to dry out completely at any point as this will alter their structure.

BOX 1 EHS user's guide

EHS is available commercially from a number of sources, including BD Biosciences (Matrigel™) and Trevigen (Cultrex® Basement Membrane Extract). EHS can also be prepared directly from EHS tumors grown as xenografts[11]. As EHS is a biological product, its components and properties, including ECM protein and growth factor concentrations, endotoxin levels and stiffness, vary between lots. It is therefore desirable to perform a series of experiments using the same lot number to minimize variation introduced by slight differences in the properties of the EHS. It is also important, when a new lot is obtained, to test whether it is appropriate for culture by performing a side-by-side comparison with cells grown in EHS from the former lot. We routinely evaluate new lots for the typical and appropriate morphogenesis of non-malignant and malignant cells along with the expression of a number of markers of interest. We also exclusively use growth factor-reduced EHS since much of our work is in the absence of serum and under defined medium conditions. Depending on the nature of the cell type and parameters to be measured, you may develop your own strategy for validation and arrive at your own EHS preferences.

BOX 2 3D on-top assay

As an alternative to the 3D embedded assay, we developed the 3D on-top assay which requires a shorter amount of time, a decreased amount of EHS, and facilitates imaging, since colonies are in a single plane. Therefore, the on-top assay is ideal for time-lapse imaging and also for *in situ* immunostaining of cell lines which form invasive stellate structures in 3D (see **Step 8, Option C**). Because less EHS is required, it is also a more cost-effective approach.

1. Thaw EHS at 4°C overnight.
2. Coat pre-chilled culture surface (e.g. dish or well) with a layer of EHS. Slowly pipette the appropriate volume (**Table 1**) directly onto surface and spread evenly with a pipette tip or

plunger of a 1 ml syringe for smaller areas or cell lifter for larger areas. Incubate for 30 min at 37°C to allow the EHS to gel (but do not let it overdry).

Culture surfaces must be pre-chilled and coated on ice to ensure even spreading of EHS. Pipette EHS slowly and directly onto culture surface to avoid formation of bubbles.

3. Trypsinize cells from a monolayer to a single cell suspension.

Use cells that are healthy and not more than 75% confluent.

4. Plate cells in half the final specified volume of cell line-appropriate medium (**Table 1**) onto the coated surface. Allow the cells to settle and attach to the EHS for 10-30 min at 37°C.

The number of cells to be plated per cm^2 of EHS surface area may need some optimization depending on the growth properties of the cell line, but we recommend the following ranges: for non-malignant cells, 0.25 x 10^5 cells/cm^2; for malignant cells, 0.175 - 0.20 x 10^5 cells/cm^2.

TROUBLESHOOTING

5. Chill remaining half volume of medium and add EHS to 10%. Gently add the EHS/medium mixture to the culture.

Medium must be thoroughly chilled before addition of EHS to ensure homogenous mixing and even deposition of EHS onto cells in culture. Pipette the drip down the side of the well to avoid disturbance of the cells or EHS gel.

6. Maintain culture for 4 days, replacing EHS/medium mixture every 2 days.

7. To perform drug response assays in 3D, see **Box 3**.

BOX 3 Drug response assays

To perform drug response assays in 3D embedded or 3D on-top cultures[7, 12, 13] (**Fig. 3** and **Supplementary Videos 1** [vehicle] and **2** [EGFR inhibitor, AG1478]), compounds may be added to the culture in one of two manners depending on their compositions:

1. Small molecule inhibitors: add to medium when cells are plated in **Step 5** of the main protocol and the 3D on-top protocol (**Box 2**). Include compound in all media changes for the duration of the culture.

2. Blocking antibodies: mix with cells before plating in **Step 4** of the main protocol and the 3D on-top protocol (**Box 2**). Include antibody in all media changes for the duration of the culture.

BOX 4 Whole culture immunostaining

When performing whole culture immunostaining in a well, the general procedure for immunostaining may be applied (see **Steps 9 - 19**), with some slight modifications:

a. Washes are performed in the well and require careful pipetting and aspiration; if cultures are not treated gently, colonies may detach from the EHS and be lost. It is advisable to check cultures frequently under a microscope to ensure they are still attached. 2X media volume is suggested for washes; for antibody incubations, 0.5X volume is sufficient.
b. Samples immunostained in a chamberslide may be mounted directly after removal of the chamber and aspiration of excess fluid.
c. Samples immunostained in a tissue culture well should be removed and mounted on a slide for long-term storage and higher quality imaging.

COMMENTS

We describe here a generalized protocol for monotypic 3D breast epithelial cell culture in the presence of lrECM, an approach which has proved to be extremely informative in our laboratory and those of others. Other workers have succeeded in culturing cell types from a wide variety of tissues using these techniques, summarized in[14]. While much of this work has been performed using EHS, other 3D substrata, such as collagen I gels, are excellent for assays of mammary gland branching morphogenesis[15], and we are watching with interest the development of additional synthetic and natural 3D substrata. Using these approaches, we hope to develop functional organotypic cultures comprised of multiple cell types, including epithelial, myoepithelial, stromal and endothelial for experimental manipulation within full context of a complex breast tissue.

ACKNOWLEDGEMENTS

The protocol described here has been the work of many members of the Bissell laboratory over many years. We apologize to those whose work could not be cited owing to space limitations and have cited reviews where possible. This work was supported by grants from the Office of Biological and Environmental Research of the Department of Energy (DE-AC03-76SF00098 and

a Distinguished Fellow Award to M.J.B.), the National Cancer Institute (CA64786 to M.J.B.; CA57621 to Zena Werb and M.J.B.), and the Breast Cancer Research Program of the Department of Defense (Innovator Award DAMD17-02-1-438 to M.J.B).

TABLE 1 Suggested volumes for 3D culture

					3D embedded		3D on-top
	# Well	Diameter (mm)	Area (cm^2)	Medium volume (µl)	EHS coat (µl)	EHS plate (µl)	EHS coat (µl)
Dish	--	60	19.5	5000	250	3600	850
Plates	6	35	9.6	2000	120	1200	500
	24	16	2.0	500	50	300	120
	48	10	0.75	200	30	150	80
	96	6	0.26	60	5	75	15
Chamber-slides	4	--	1.8	500	50	300	120
	8	--	0.8	200	30	150	90

Note that the volumes of EHS to be plated are not directly proportional to surface area. The viscosity of EHS causes it to form a meniscus in the well, the effect of which increases with decreasing well size. As a corollary, for 3D on-top cultures, the smaller the well size, the less flat the plane of plating will be.

FIGURE LEGENDS

Figure 1 Breast epithelial cell morphology in differing culture conditions. (**a**) Schematic of non-malignant breast epithelial cells grown as a monolayer on tissue culture plastic (left), in the 3D embedded assay (middle), and in the 3D on-top assay (right). (**b**) Phase contrast images of (top) non-malignant HMT-3522 S1 cells grown in the 3 different culture conditions and (bottom) malignant HMT-3522 T4-2 cells grown in the same conditions. Scale bar, 50 μm.

Figure 2 Breast cancer cell lines in 3D culture. Phase contrast images (left panel) and confocal cross-sections of Phalloidin staining of F-actin (right panel) of (**a**) BT474; (**b**) SKBR3; and (**c**) MDA-MB 231 cell lines grown for 4 days in the 3D on-top assay. In (**a**) and (**b**), colonies were completely extracted from the gel for immunostaining; in (**c**), colonies were immunostained in the gel. Scale bars, 100 μm (left panel) and 50 μm (right panel).

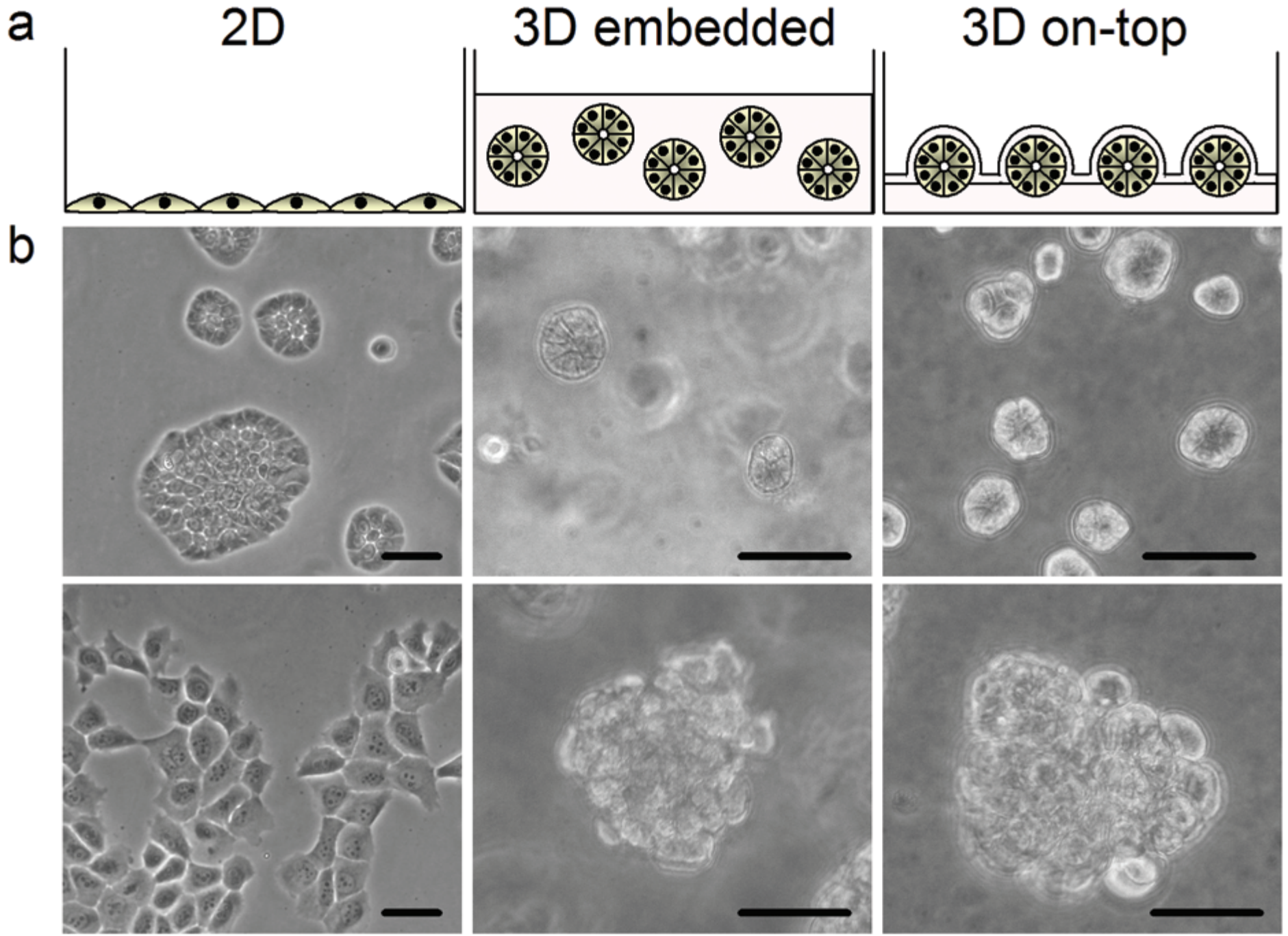
Figure 3 3D drug response assay. HMT-3522 S1 (**a**), HMT-3522 T4-2 (**b**), and HMT-3522 T4-2 treated with an EGFR inhibitor, AG1478 (**c**) were cultured in the 3D on-top assay for 4 days. Colonies were then extracted and immunostained against α6 integrin (green) and β-catenin (red). Nuclei were counterstained with DAPI (blue). Confocal sections through the centers of the colonies are shown. Scale bar, 20 μm.

Supplemental Video 1 Time lapse movies of HMT-3522 T4-2 cells treated with vehicle and cultured in the 3D on-top assay for 4 days. Phase contrast images were taken at 1 hour intervals.

Supplemental Video 2 Time lapse movies of HMT-3522 T4-2 cells treated with an EGFR inhibitor, AG1478, and cultured in the 3D on-top assay for 4 days. Phase contrast images were taken at 1 hour intervals.

REFERENCES

1. Bissell, M. J., Radisky, D. C., Rizki, A., Weaver, V. M. & Petersen, O. W. The organizing principle: microenvironmental influences in the normal and malignant breast. Differentiation 70, 537-46 (2002).
2. Bissell, M. J., Kenny, P. A. & Radisky, D. C. Microenvironmental regulators of tissue structure and function also regulate tumor induction and progression: the role of extracellular matrix and its degrading enzymes. Cold Spring Harb Symp Quant Biol 70, 343-56 (2005).
3. Petersen, O. W., Ronnov-Jessen, L., Howlett, A. R. & Bissell, M. J. Interaction with basement membrane serves to rapidly distinguish growth and differentiation pattern of normal and malignant human breast epithelial cells. Proc Natl Acad Sci U S A 89, 9064-8 (1992).
4. Anders, M. et al. Disruption of 3D tissue integrity facilitates adenovirus infection by deregulating the coxsackievirus and adenovirus receptor. Proc Natl Acad Sci U S A 100, 1943-8 (2003).
5. Bissell, M. J., Rizki, A. & Mian, I. S. Tissue architecture: the ultimate regulator of breast epithelial function. Curr Opin Cell Biol 15, 753-62 (2003).
6. Wang, F. et al. Phenotypic reversion or death of cancer cells by altering signaling pathways in three-dimensional contexts. J Natl Cancer Inst 94, 1494-503 (2002).
7. Wang, F. et al. Reciprocal interactions between beta1-integrin and epidermal growth factor receptor in three-dimensional basement membrane breast cultures: a different perspective in epithelial biology. Proc Natl Acad Sci U S A 95, 14821-6. (1998).
8. Kleinman, H. K. et al. Isolation and characterization of type IV procollagen, laminin, and heparan sulfate proteoglycan from the EHS sarcoma. Biochemistry 21, 6188-93 (1982).
9. Liu, H., Radisky, D. C., Wang, F. & Bissell, M. J. Polarity and proliferation are controlled by distinct signaling pathways downstream of PI3-kinase in breast epithelial tumor cells. J Cell Biol 164, 603-12 (2004).
10. Sambrook, J., Fritsch, E. F. & Maniatis, T. Molecular Cloning: A Laboratory Manual (Cold Spring Harbor Laboratory Press, New York, 1989).
11. Kibbey, M. C. Maintenance of the EHS sarcoma and matrigel preparation. Methods in Cell Science 16, 227-230 (1994).
12. Weaver, V. M. et al. Reversion of the malignant phenotype of human breast cells in three-dimensional culture and in vivo by integrin blocking antibodies. J Cell Biol 137, 231-45. (1997).
13. Weaver, V. M. et al. beta4 integrin-dependent formation of polarized three-dimensional architecture confers resistance to apoptosis in normal and malignant mammary epithelium. Cancer Cell 2, 205-16 (2002).
14. Kleinman, H. K. & Martin, G. R. Matrigel: basement membrane matrix with biological activity. Semin Cancer Biol 15, 378-86 (2005).
15. Fata, J. E., Werb, Z. & Bissell, M. J. Regulation of mammary gland branching morphogenesis by the extracellular matrix and its remodeling enzymes. Breast Cancer Res 6, 1-11 (2004).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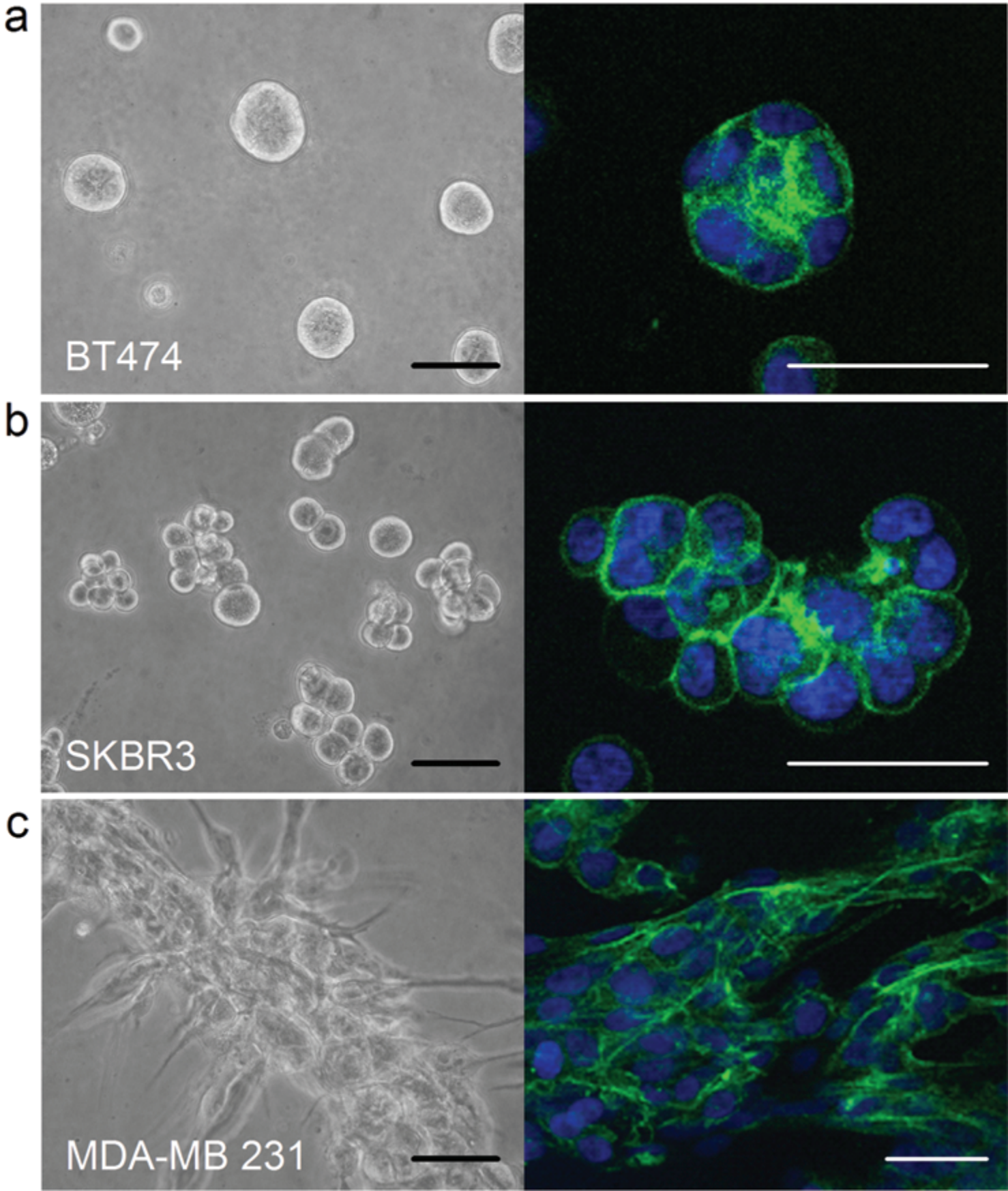
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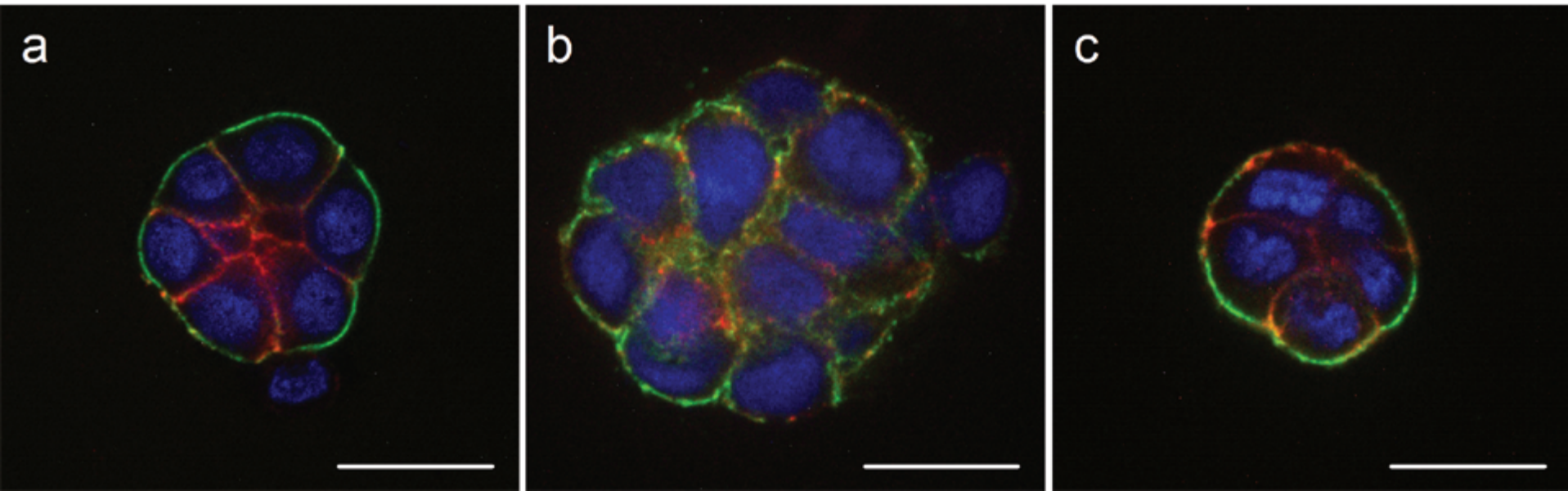
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