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Establishment and analysis of three-dimensional (3D) organoids derived from patient prostate cancer bone metastasis specimens and their xenografts

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TITLE:

Establishment and Analysis of Three-Dimensional (3D) Organoids Derived from Patient Prostate Cancer Bone Metastasis Specimens and their Xenografts

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SUMMARY:

Three-dimensional cultures of patient BMPC specimens and xenografts of bone metastatic prostate cancer maintain the functional heterogeneity of their original tumors resulting in cysts, spheroids and complex, tumor-like organoids. This manuscript provides an optimization strategy and protocol for 3D culture of heterogeneous patient derived samples and their analysis using IFC.

ABSTRACT:

Three-dimensional (3D) culture of organoids from tumor specimens of human patients and patient-derived xenograft (PDX) models of prostate cancer, referred to as patient-derived

organoids (PDO), are an invaluable resource for studying the mechanism of tumorigenesis and metastasis of prostate cancer. Their main advantage is that they maintain the distinctive genomic and functional heterogeneity of the original tissue compared to conventional cell lines that do not. Furthermore, 3D cultures of PDO can be used to predict the effects of drug treatment on individual patients and are a step towards personalized medicine. Despite these advantages, few groups routinely use this method in part because of the extensive optimization of PDO culture conditions that may be required for different patient samples. We previously demonstrated that our prostate cancer bone metastasis PDX model, PCSD1, recapitulated the resistance of the donor patient's bone metastasis to anti-androgen therapy. We used PCSD1 3D organoids to characterize further the mechanisms of anti-androgen resistance. Following an overview of currently published studies of PDX and PDO models, we describe a step-by-step protocol for 3D culture of PDO using domed or floating basement membrane (e.g., Matrigel) spheres in optimized culture conditions. In vivo stitch imaging and cell processing for histology are also described. This protocol can be further optimized for other applications including western blot, co-culture, etc. and can be used to explore characteristics of 3D cultured PDO pertaining to drug resistance, tumorigenesis, metastasis and therapeutics.

INTRODUCTION:

Three-dimensional cultured organoids have drawn attention for their potential to recapitulate the in vivo architecture, cellular functionality and genetic signature of their original tissues[1-5]. Most importantly, 3D organoids established from patient tumor tissues or patient derived xenograft (PDX) models provide invaluable opportunities to understand mechanisms of cellular signaling upon tumorigenesis and to determine the effects of drug treatment on each cell population[6-13]. Drost et al.[5] developed a standard protocol for establishment of human and mouse prostate organoids, which has been widely adopted in the field of urology. In addition, significant effort has been dedicated for further characterization of 3D organoids and to understand the detailed mechanisms of tumorigenesis and metastasis[4,12,14,15]. In addition to the previously established and widely accepted protocol for 3D organoids cultures, we describe here a step-by-step protocol for the 3D culture of PDO using three different doming methods in optimized culture conditions.

In this manuscript, 3D organoids were established as an ex vivo model of bone metastatic prostate cancer (BMPC). The cells used for these cultures came from the Prostate Cancer San Diego (PCSD) series and were derived directly from patient prostate cancer bone metastatic tumor tissues (PCSD18 and PCSD22) or patient derived xenograft (PDX) tumor models (samples named PCSD1, PCSD13, and PCSD17). Because spontaneous bone metastasis of prostate cancer cells is rare in genetically engineered mouse models[16], we used direct intra-femoral (IF) injection of human tumor cells into male $Rag2^{-/-}\gamma c^{-/-}$ mice to establish the PDX models of bone metastatic prostate cancer[17].

Once 3D organoids are established from heterogeneous patient tumor cells or patient derived xenografts, it is essential to confirm their identity as prostate tumor cells and to determine their phenotypes in the 3D organoid cultures. Immunofluorescence chemistry (IFC) allows the visualization of protein expression in situ in each cell, often indicating the potential functions

for specific cell populations[2,4]. In general, IFC protocols for a vast majority of samples including tissues and cells are straightforward and fully optimized. However, the cell density and number of organoids can be significantly lower than that of conventional culture. Therefore, the IFC protocol for organoids requires additional steps to ensure proper processing and embedding in paraffin for all organoids in the samples. We describe additional steps for an agarose pre-embedding process and tips to label the location of sectioned organoids on the slide that increases the success rate of IFC on organoids especially when the samples of organoids have lower cell density than desired.

PROTOCOL:

This study was carried out in strict accordance with the recommendations in the Guide for the University of California San Diego (UCSD) Institutional Review Board (IRB). IRB #090401 Approval was received from the UCSD Institutional Review Board (IRB) to collect surgical specimen from patients for research purposes. An informed consent was obtained from each patient and a surgical bone prostate cancer metastasis specimen was obtained from orthopedic repair of a pathologic fracture in the femur. Animal protocols were performed under the University of California San Diego (UCSD) animal welfare and Institutional Animal Care and Use Committee (IACUC) approved protocol #S10298. Cells from mechanically and enzymatically dissociated patient tumor tissue were intra-femorally injected into 6 to 8 week old male *Rag2*$^{-/-}$*;*$\gamma_c^{-/-}$ mice as previously described[17]. Xenograft tumor volume was determined using an *in vivo* bioluminescence imaging system and caliper measurements. Upon tumor growth up to 2.0 cm (the maximal allowable size approved by IACUC), the tumor was harvested for 3D organoids establishment.

NOTE: **Figure 1** shows the workflow for establishing 3D Organoids and a protocol number for each step of the experimental procedures.

1. Processing of patient derived xenograft (PDX) tumor tissues

NOTE: This is an initial step for organoid establishment for a tumor derived from a xenograft mouse model. This protocol is adapted from a previous publication by Drost et al.[5] and we have modified the media conditions to include serum supplementation to organoid media.

1.1. Process the tumor specimen as described below.

1.1.1. Mince tumor samples to 1-3 mm^3 sized pieces and digest the samples with 10 mL of cell dissociation solution for 45 min at room temperature.

1.1.2. To terminate digestion, add 20 mL of DMEM complete media to the samples.

1.1.3. Filter the suspension through a 70 μm cell strainer. Use a sterile plunger flange to push any leftover tissue on the top of 70 μm cell strainer.

1.1.4. Centrifuge at 300 x *g* for 5 min at 4 °C.

1.1.5. Wash the cell pellet three times with fresh adDMEM complete media. Match the volume of media for wash and resuspension with the volume of media suggested in **Table 3**. For example, for a 24 well plate culture condition, the volume for the wash should be 500 µL.

NOTE: As shown in **Table 3**, the protocol is applicable to different culture conditions.

1.1.6. Determine final cell counts using Trypan blue dye and a hemocytometer.

1.1.7. After obtaining cell counts, re-suspend cell pellet in 80 µL of 2% FBS in PBS per 2 x 10^6 tumor cells.

1.1.8. Add 20 µL of Mouse Cell Depletion Cocktail per 2 x 10^6 tumor cells. Mix well and incubate for 15 min at 2-8 °C.

1.1.9. Adjust the volume to 500 µL with 2% FBS in PBS buffer per 2 x 10^6 tumor cells.

NOTE: Up to 1 x 10^7 tumor cells in 2.5 mL of cell suspension can be processed on one LS column.

1.1.10. Load the LS columns on the magnetic column separator and place a 15 mL conical tube on a rack underneath to collect the flow-through.

1.1.11. Rinse each column with 3 mL of 2% FBS in PBS buffer. Discard the conical tube with the wash flow-through and replace with a new, sterile 15 mL conical tube.

1.1.12. Add the cell suspension (up to 2.5 mL of 1 x 10^7 tumor cells) onto the column. Collect flow-through that will be the enriched with human tumor cells.

1.1.13. Wash the column twice with 1 mL of 2% FBS in PBS buffer.

NOTE: It is important to perform wash steps as soon as the column is empty. Also, try to avoid forming air bubbles.

1.2. Aliquot the appropriate volume of cell suspension to a 1.5 mL tube for the desired culture set up (**Table 3**).

1.3. Centrifuge the 1.5 mL tube at 300 x *g* and 4 °C for 5 min.

1.4. Carefully remove and discard the supernatant.

2. Processing of patient primary tumor tissues

NOTE: This is an initial step for organoid establishment.

2.1. Follow all of step 1 **except** the mouse cell depletion process, which is not necessary for processing of patient primary tumor tissues.

3. Forming an attached round dome on the plate

NOTE: This manuscript describes three ways to make a dome from a mixture of the cell pellet and the basement membrane (e.g., Matrigel) as shown in **Figure 1** and **Figure 2**. In Steps 2-4, the cells and the basement membrane should be kept on ice to prevent solidification of the basement membrane.

3.1. Resuspend the cell pellet in the appropriate volume of basement membrane (e.g., 40 µL) for a 24 well plate set up (**Table 1**).

3.2. Pipette up and down gently to ensure that the cells are re-suspended well in the basement membrane.

3.3. Pipette the appropriate volume (**Table 3**) of the cell-basement membrane mixture (and optional 10 µL of adDMEM complete media) into the center of the pre-warmed issue culture plate.

3.4. Invert the plate and immediately place the plate upside down in the CO_2 cell culture incubator set at 5% CO_2, 37 °C for 15 min. This prevents cells from settling and adhering to the plate bottom while allowing the basement membrane to solidify.

3.5. Pipette the appropriate volume (**Table 3**) of pre-warmed medium containing 10 µM Y-27632 dihydrochloride into each well.

3.6. Place the plate right side up inside the CO_2 cell culture incubator (5% CO_2, 37 °C).

3.7. Change the media every 3-4 days. After 5-7 days, use culture medium without 10 µM Y-27632 dihydrochloride to maintain the cultures.

4. Forming a floating dome from an attached round dome on the plate

4.1. After step 3.7, detach the dome using a cell scraper.

5. Forming floating beads

NOTE: This protocol is named as floating beads since the mixture of basement membrane, media, and organoids look like beads.

5.1. Cut a 2 inch x 4 inch piece of paraffin film.

5.2. Place the paraffin film on the top of the divots of an empty tip-holding rack from a 1000 µL plastic pipette tip box.

5.3. Gently press down on the paraffin film to trace the divots using a gloved index finger but without breaking through the paraffin film.

5.4. Spray the paraffin film with 70% ethanol and turn on the UV lamp in the cell culture hood to sterilize the prepared paraffin film for at least 30 min.

5.5. Prepare a mixture of cells and 20 µL of basement membrane. Seeding density can be 50,000 - 250,000 cells per dome.

5.6. Pipette the mixture of cells processed from step 1 or 2, and 20 µL of basement membrane into the mold of the divot formed in the prepared paraffin film.

5.7. Resuspend the cell pellet in basement membrane and pipette the cell suspension in the prepared paraffin filmed divots.

5.8. Place the solidified beads and paraffin film into a 6-well plate. One well in a 6-well plate can fit up to 5 beads.

5.9. Pipette 3-5 mL of pre-warmed medium containing 10 µM Y-27632 dihydrochloride into each well while gently brushing beads off of the paraffin film.

NOTE: As a minimum volume, 3 mL is recommended. For a maximum number of beads (N=5) per well, 5 mL of medium is recommended.

5.10. Place the plate inside a CO_2 incubator (5% CO_2, 37 °C).

5.11. Change the organoid media every 3-4 days. After 5-7 days, use culture medium without 10 µM Y-27632 dihydrochloride to maintain the cultures.

6. In vivo organoids image stitching using microscope[18]

NOTE: Certain microscopes are unable to reach the outer perimeter of the cell plate (edge wall); therefore, we suggest using the wells close to the perimeter of the cell plate when image stitching.

6.1. Place the cell culture plate in an upward position into the plate holder in the Keyence microscope.

6.2. Place the lens on the center of the target dome.

6.3. Set up the automatic stitching process by selecting number of the frames. For examples, 3 x 3 or 5 x 5 can be chosen to generate 9 images or 25 images total.

6.4. Press the capture button to initiate imaging process.

6.5. Open the image viewer software and load a group of images taken by step 4.

6.6. Click **Image Stitching** to create a high-resolution stitched image.

NOTE: Capturing of serial 9 or 25 images can be performed either by manual or automatic set up to focus the cells.

7. Organoid processing for histology: the agarose spin down method

NOTE: This protocol is adapted from a previous publication by Vlachogiannis et al.[7]. We have added a step involving agarose embedding to successfully embed all populations of organoids.

7.1. Remove existing media from the well. Be careful not to aspirate the basement membrane domes.

7.2. Add an equal (equal to the volume of media removed from step 1) volume of cell recovery solution and incubate for 60 min at 4 °C.

7.3. Dislodge the basement membrane dome using a pipette and crush the basement membrane dome using a pipet tip. Collect the dissociated dome and cell recovery solution in a 1.5 mL tube.

7.4. Centrifuge at 300 x *g* and 4 °C for 5 min.

7.5. Remove the supernatant (cell recovery solution). Save all supernatants in separate tubes until the end when the presence of organoids is confirmed in the final pelleting step.

7.6. Add desired volume (**Table 3**) of cold PBS and gently pipette up and down to mechanically disturb pellet.

7.7. Centrifuge at 300 x *g* and 4 °C for 5 min.

7.8. Remove the supernatant (PBS).

7.9. Fix the pellet in a matched volume (e.g., 500 µL for one pellet from the 24 well plate culture condition, **Table 3**) of 4% PFA for 60 min at room temperature.

7.10. Following fixation, centrifuge at 300 x g and 4 °C for 5 min.

7.11. Remove the supernatant (PFA).

7.12. Wash with matched volume (e.g., 500 µL for one pellet from the 24 well plate culture condition, **Table 3**) of PBS and centrifuge at 300 x g and 4 °C for 5 min.

7.13. Prepare warm agarose (2% agarose in PBS).

NOTE: Here, cell pellets for frozen sections can be directly re-suspended in 200 µL of OCT compound without further steps in Protocol 7.

7.14. Re-suspend the cell pellet in 200 µL of agarose (2% in PBS).

7.14.1. Immediately after adding agarose, gently detach the cell pellet from the wall of the 1.5 mL tube using the 25 G needle attached to 1 mL syringe. As shown in **Figure 3**, if the cell pellet is not physically detached from the wall of the 1.5 mL tube, then there is a risk of losing all or part of the cell pellet during the agarose embedding process.

7.15. Wait until the 2% agarose in PBS is completely solidified.

7.16. Detach the solidified agarose block from the 1.5 mL tube using a 25 G needle attached to the 1 mL syringe.

7.17. Transfer the detached agarose block containing the cell pellet to a new 1.5 mL tube.

7.18. Fill the tube with 70% EtOH and proceed further using the conventional protocol for tissue dehydration and paraffin embedding.

8. Histology and Immunofluorescent cytochemistry (IFC) of organoids

8.1. Select the slide(s) for histology or IFC.

8.2. Before initiating the staining process, find out where the cells are located on the slide and draw a circle around the cells on the slide using a marker.

8.3. Draw the perimeter around the edge or boarder of the slide and where circles are located on the slide in a laboratory notebook to record their locations.

8.4. Perform desired staining.

NOTE: During this process, marked circles disappear since regular maker is not resistant to the chemicals. Even some histology permanent markers may be erased during staining process.

8.5. After the staining process, place the slide over the drawing in the laboratory notebook to find the locations of cells on the slide.

REPRESENTATIVE RESULTS:

3D organoids were successfully established from a patient derived xenograft (PDX) model of bone metastatic prostate cancer (BMPC) as well as directly from patient bone metastatic prostate cancer tissue (**Figure 4**). Briefly, our PDX models of BMPC were established by intra-femoral (IF) injection of tumor cells into male $Rag2^{-/-}$ $c^{-/-}$ mice and then PDX tumors were harvested and processed as described in this manuscript. As shown in **Figure 4**, PDX tumor tissues from the PCSD series resulted in 3D organoids with differential phenotypes that manifested as cysts, spheroids and higher complexity organoids that formed using this protocol.

A stitched image from 25 high resolution 10x magnification images showed an entire dome of basement membrane and organoids (**Figure 5**). Using image analysis software, one has the option to sort out the cells or cell clusters that are larger than a certain size or to manually select spheroids or cysts.

The steps for agarose embedding of the organoid cultures and tips to label the location of sectioned organoids on the slide increase the success of performing IFC on the organoids especially when the samples of organoids have a lower cell density than desired. **Figure 6** shows an example of IFC on a 5 μm thick paraffin section of organoids targeting cytokeratin 5 (CK5, basal epithelial cell marker), cytokeratin 8 (CK8, luminal epithelial cell marker) and DAPI.

FIGURE AND TABLE LEGENDS:

Figure 1. Establishment and characterization for 3D cultured organoids derived either from patient tumor tissues or patient derived xenograft (PDX) tumor tissues.

Figure 2. Methodologies to form a mixture of cell pellets and basement membrane. An attached dome (**a**), a floating dome (**b**) and floating beads (**c**).

Figure 3. A key step for successful agarose embedding. A process to detach the cell pellet from the wall of the 1.5 mL tube.

Figure 4. Differential phenotypes of organoids from a series of PCSD PDX tumors, which includes single cells, cysts, spheroids and higher complexity organoids.

Figure 5. Example of a raw stitched image from total of 25 high resolution images and its application for a follow up analysis to count the number of cells or measure the area of cells/cell clusters.

Figure 6. Image showing CK5 and CK8 IFC stained PCSD1 organoids (5 μm thickness of paraffin section).

Table 1. adDMEM/F12 +/+/+ Preparation. This table has been previously published by Drost et al.[5].

Table 2. Components for C/S Human Media + 10 % FBS. This table has been previously published by Drost et al.[5].

Table 3. Seeding density, basement membrane volume, and medium volume needed for one dome. This table is modified from a previous publication by Drost et al.[5].

DISCUSSION:

3D organoids derived from patient bone metastasis prostate cancer cells are still relatively rare. Here, we describe strategies and further optimized protocol to successfully established serial 3D patient derived organoids (PDOs) of BMPC. In addition, protocols are described to secure the organoids in samples with lower cell density for IFC and IHC analysis. Differential phenotypes in the form of cyst, spheroids and more complex organoids indicate that this protocol provides culture conditions that allows for heterogonous tumor cell populations in patients to retain their cellular phenotype and structure. Thus, the culture conditions described here, which were adapted from Drost et al.[5] modified with FBS serum supplementation, has been shown to be optimal for the cultures of patient BMPC-derived cells such that they retain their intra-subject and inter-subject heterogeneity (manuscript in preparation).

Our optimized protocol is practical for an experimental set up with multiple endpoints from limited starting material from primary or PDX tumor tissues to set up 3D organoids cultures. In general, establishment of 3D organoids from patient primary tumor tissues is less successful than establishment of 3D organoids from PDX tumor tissues. Therefore, it is recommended to have a higher seeding density (up to four times higher) of cells for the establishment of 3D organoids from patient primary tumor tissues than from PDX tumors). One method to increase cell yield during tumor tissue processing is to recover any tumor tissue leftover on the top of 70 μm cell strainer by passing the filtered cell suspension through the strainer again.

Three different methods to form a 3D organoid culture (**Figure 2**) can be used and easily adapted depending on the follow up endpoint analysis. For example, if one desired to have the stitched images after establishing 3D organoids, an attached dome is highly recommended since the image stitching process requires a serial movement of microscopic objective to acquire the desired number of images (either 9 or 25). This process results in a subtle vibration to the holder of cell culture plate. Both types of the floating dome will freely move due to the vibration while acquiring the image. In addition, both types of floating domes can be easily transferred from one well to the another, making them suitable for co-culture with adherent cells after setting up 3D organoid cultures and the adherent cells in the separate wells. The attached dome and floating dome released from being attached have been shown to retain organoids for up to 10 weeks. Interestingly, a floating dome from the floating beads method has been shown to retain organoids for up to 4 months. The floating beads method to produce basement membrane spheres introduced in our protocol involves more steps and skills but can be considered for a long-term culture. In parallel, despite their use for establishment of 3D organoids from different tumors and metastasis, the role of the doming method and the basement membrane shape on organoids formation have not been deeply determined. In that

sense, the paraffin film method can be considered for use when the other two methods have been unsuccessful.

For all three different methods, this protocol recommends using a lower volume of media and a higher volume of basement membrane for the resuspension process because the domes will last longer in culture. A dome is formed with a 1:4 ratio of media and basement membrane. Of note, this method increases the formation of bubbles while pipetting the cell suspension up and down since the added volume of media does not circumvent the viscosity of the basement membrane. Therefore, it is recommended to set up 1.5 mL tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 mL tube. In doing so, the formation of bubbles in one tube for one dome will not serially affect other domes. If the duration of the culture condition has been previously optimized and is known to be short (up to a month), the basement membrane can be diluted more to circumvent the viscosity issue of the basement membrane. A cell pellet resuspension for several wells can be prepared in one tube, utilizing a higher volume of media to the desired volume of basement membrane to minimize cell loss and variability.

To minimize bubble formation during the pipetting process, pipette a lower volume than the actual volume of the cells, media and basement membrane mixture. For example, for a dome within 24 well plates, 40 µL of basement membrane and up to 10 µL of adDMEM complete media are mixed with the cell pellet. In this case, the pipette can be set to handle a volume of 40 µL instead of 50 µL while pipetting up and down in order to minimize bubble formation. For this second method, the cell pellet resuspension for several wells can be prepared in one tube to minimize cell loss and variability. The liquid status of commercially available basement membrane is often variable. Therefore, new batches of basement membrane need to be tested for the doming process. If the basement membrane is more viscous than usual, then up to additional 10 µL of adDMEM complete media (details written in step 1) can be added to dilute the mixture of cell pellet and basement membrane.

Here, we present a protocol to establish, image and process cultured 3D organoid with detailed tips for successful completion of the desired procedures. Culture media for 3D organoids introduced in our manuscript is specifically for prostate-derived cells. However, other details described in our protocol are applicable to different types of tumor tissues and metastatic sites.

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DISCLOSURES:
Sanghee Lee and Christina A.M. Jamieson are the guest editors of JoVE Methods Collection.

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Figure 1

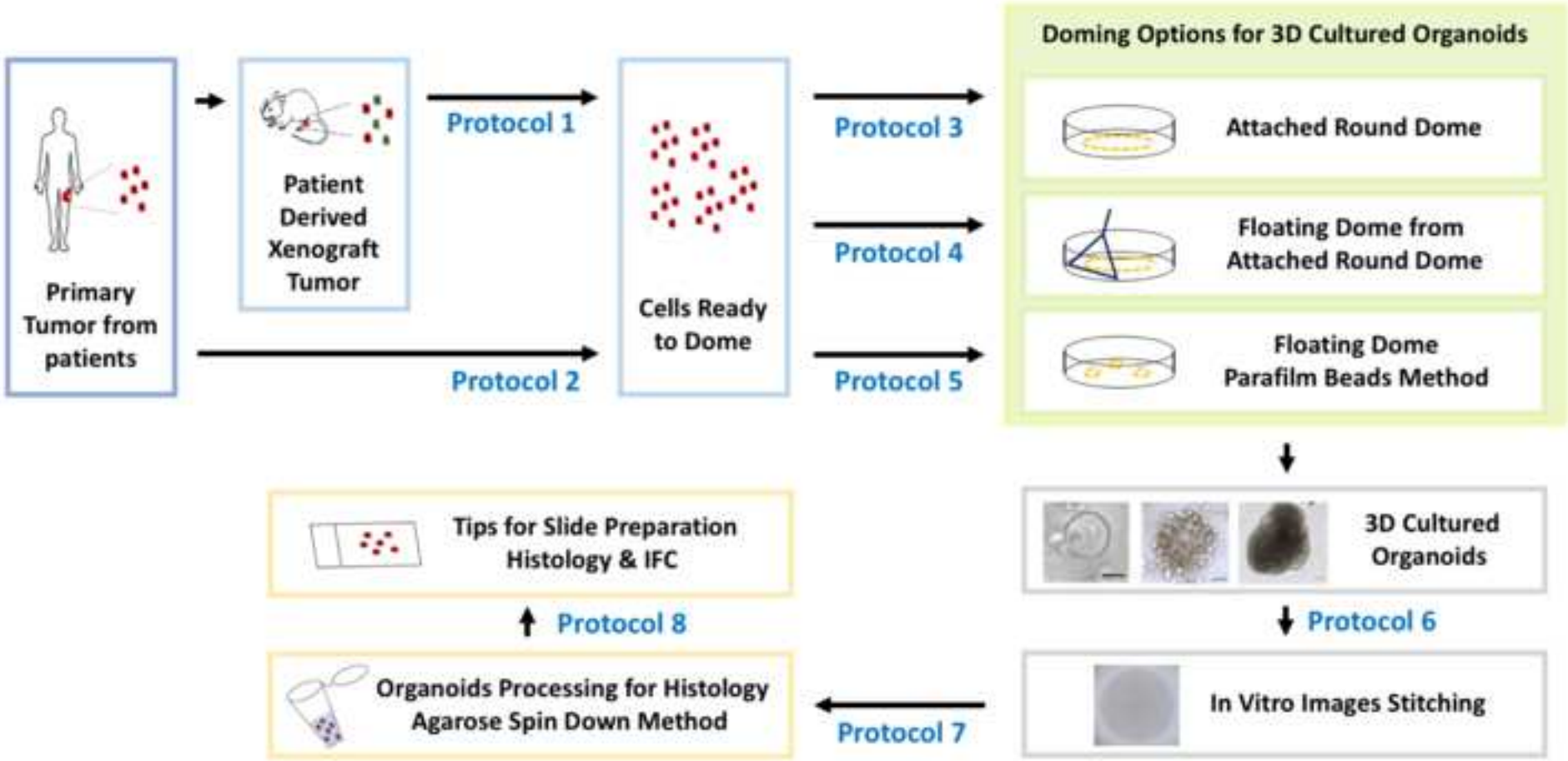
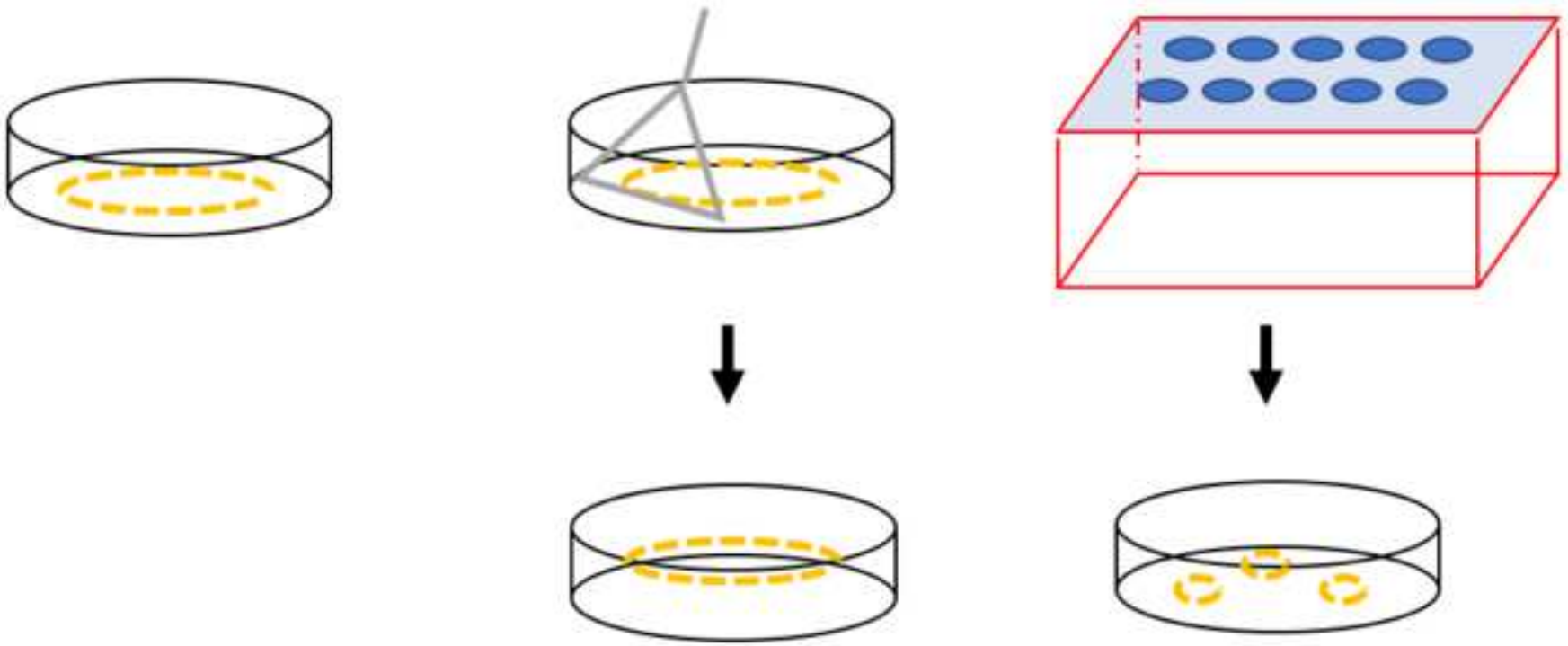


Figure 2



(a) Attached Dome

(b) From Attached Dome to Floating Dome

(c) Parafilm Beads

Figure 3

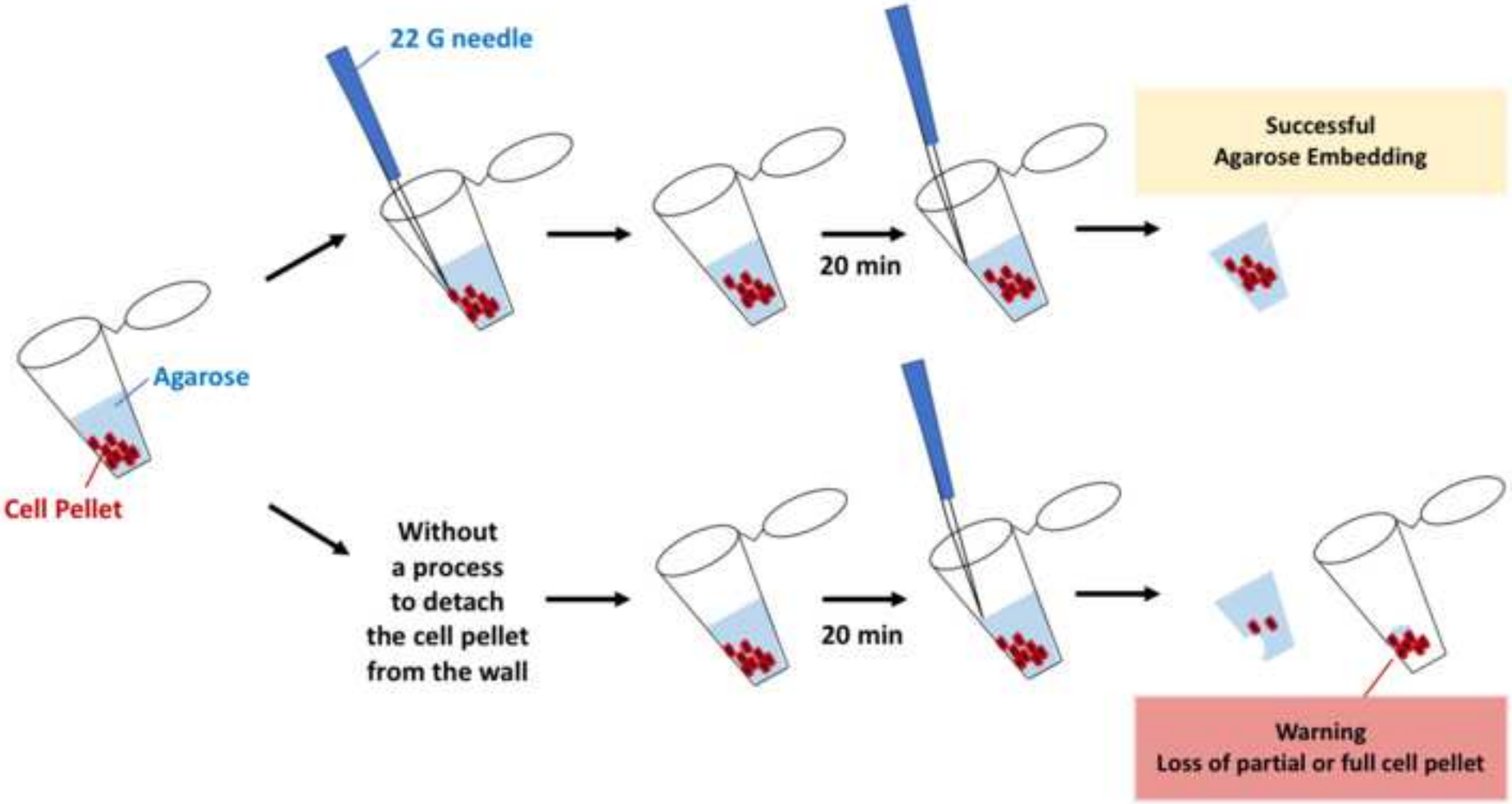


Figure 4

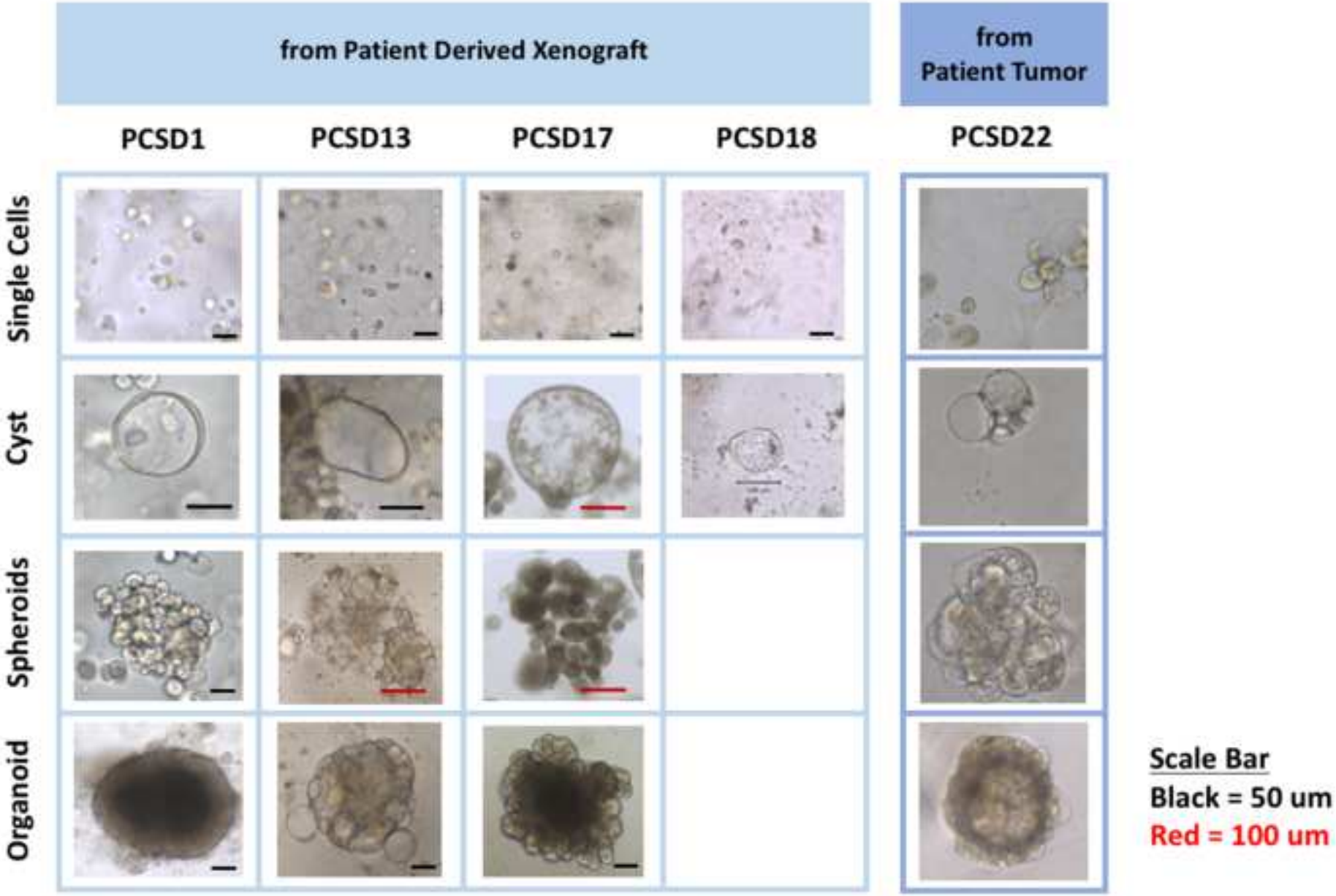


Figure 5

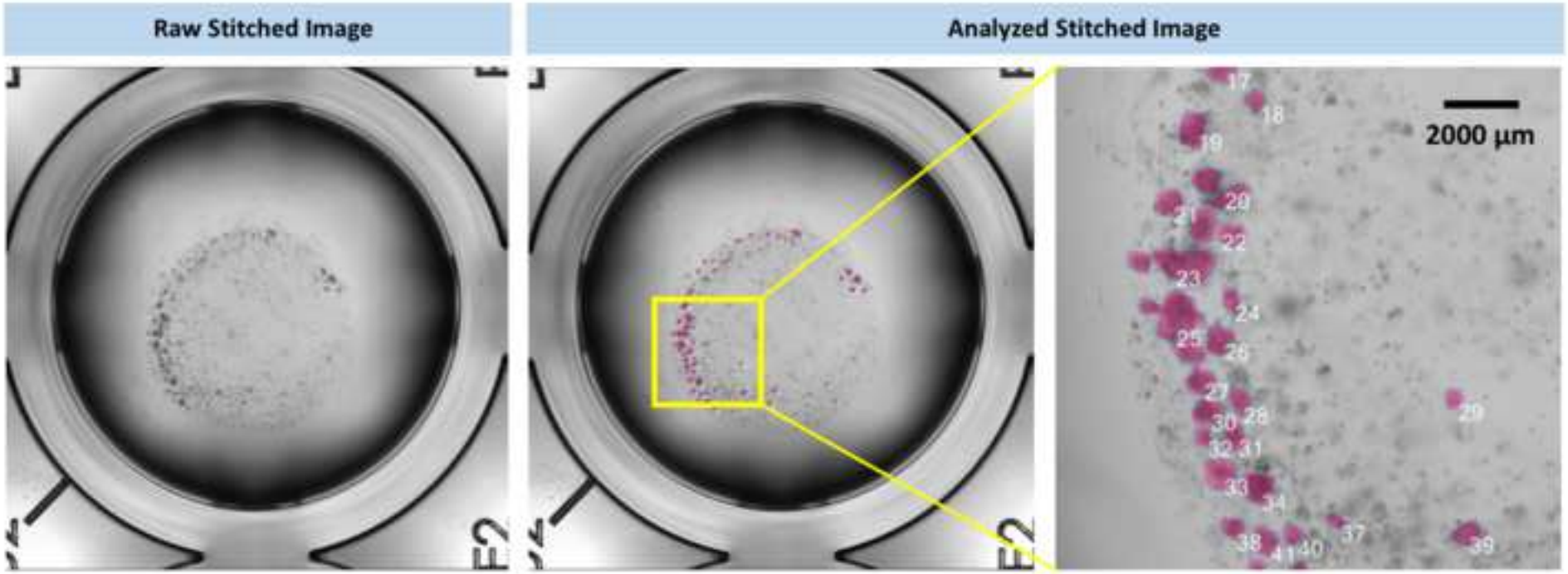


Figure 6

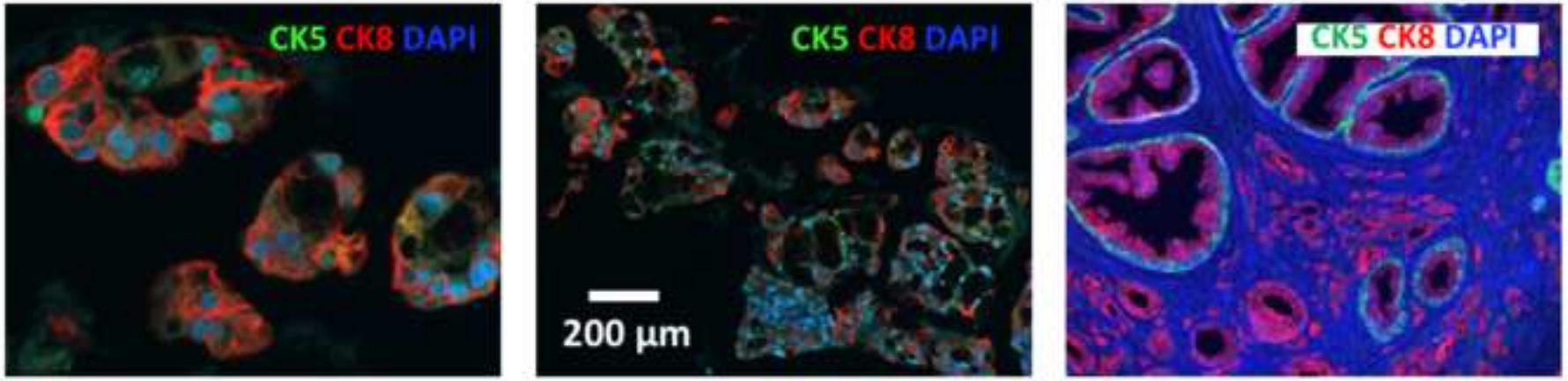


Table 1

Stock Component	Final Concentration	Vol (mL) needed for 500 ml solution
1000 mM HEPES	10 mM	5
200 mM Glutamax	2 mM	5
100x Pen-Strep	1x	5
adDMEM/F12 +/+/+		485

Table 2

Stock Component	Final Concentration	Vol (µL) needed for 50 mL solution	Vol (µL) needed for 25 mL solution
50x B27	50x Diluted	1000	500
500 mM N-Acetylcysteine	1.25 mM	125	62.5
0.5 mg/mL EGF	5 ng/mL	0.5	0.3
100 ug/mL Noggin	100 ng/mL	50	25
R-Spondin 1	10% conditioned medium	5000	2500
5 mM A83-01	500 nM	5	2.5
0.1 mg/mL FGF10	10 ng/mL	5	2.5
50 µg/mL FGF2	5 ng/mL	5	2.5
10 mM Prostaglandin E2	1 µM	5	2.5
1M Nicotinamide	10 mM	500	250
30 mM SB202190	10 µM	16.7	8.4
FBS	10%	5000	2500
1 µM DHT	1 nM	50	25
adDMEM/F12 +/+/+		Bring up to 50 ml	Bring up to 25 mL
100 mM Y-27632 Dihydrochloride	10 µM	5	2.5

Table 3

Culture Plate	Seeding Density (cells)	ment Membrane Volume	Medium (µL)
48 well	25,000-50,000	20	250
24 well	50,000-250,000	40	500
6 well	50,000-250,000	40	2,000

Table of Materials

Click here to access/download;Table of Materials;JoVE60307 Revision Materials and Reagents - Jamieson.xlsx

Name of Material/ Equipment	Company	Catalog Number
1 mL Pipettman	Gilson	F123602
1 mL Syringe	BD Syringe	329654
1.5 mL tube	Spectrum Lab Products	941-11326-ATP083
25G Needle	BD PrecisionGlide Needle	305122
4% Paraformaldehyde (PFA)	Alfa Aesar	J61899
70% Ethanol (EtOH)	VWR	BDH1164-4LP
A83-01	Tocris Bioscience	2939
Accumax	Innovative Cell Technologies, Inc.	AM105
adDMEM	Life Technologies	12634010
Agarose	Lonza	50000
Antibody -for Cytokeratin 5	Biolegend	905901
Antibody for Cytokeratin 8	Biolegend	904801
B27	Life Technologies	17504044
Bioluminescence imaging system, IVIS 200	Perkin Elmer Inc	IVIS 200
Cell Culture Plate - 24 well	Costar	3524
Cell Culture Plate - 48 well	Costar	3548
Cell Culture Plate - 6 well	Costar	3516
Cell Dissociation Solution, Accumax	Innovative Cell Technologies, Inc.	AM105
Cell Recovery Solution	Corning	354253
Cell Scraper	Sarstedt	83.180
Cell Strainer	Falcon (Corning)	352350
CO2 incubator	Fisher Scientific	3546
DAPI	Vector Vectashield	H-1200
DHT	Sigma-Aldrich	D-073-1ML
dPBS	Corning/Cellgro	21-031-CV
EGF	PeproTech	AF-100-15
FBS	Gemini Bio-Products	100-106
FGF10	PeproTech	100-26
FGF2	PeproTech	100-18B
Forceps	Denville Scientific	S728696
Glutamax	Gibco	35050-061
HEPES	Gibco	15630-080
LS Columns	Miltenyi	130-0420401
Magnetic Column Seperator: QuadroMACS Separator	Miltenyi	130-090-976
Marker	VWR	52877-355
Matrigel (Growth Factor Reduced)	Mediatech Inc. (Corning)	356231
Matrigel (High Concentration)	BD (Fisher Scientific)	CB354248
Microscope Imaging Software, Keyence	BZ-X800 (newest software) BZ-X700 (old software)	
Microscope, Keyence	BZ-X700 (model 2016-2017)/BZ-X710 (model 2018-2019)	
Mouse Cell Depletion Kit	Miltenyi	130-104-694
N-Acetylcysteine	Sigma-Aldrich	A9165-5G
Nicotinamide	Sigma-Aldrich	N0636-100G
Noggin	PeproTech	120-10C
OCT Compound	Tissue-Tek	4583
Parafilm	American National Can	N/A
Pen-Strep	Mediatech Inc. (Corning)	30-002-CI-1
Pipette tipes for 1 mL (Blue Tips)	Fisherbrand Redi-Tip	21-197-85
Plunger (from 3 mL syringe)	BD Syringe	309657
Prostaglandin E2	Tocris Bioscience	2296
R-Spondin 1	Trevigen	3710-001-01
SB2021190	Sigma-Aldrich	S7076-25MG
Small Table Top Centrifuge	ThermoFisher Scientific	75002426
Water Bath	Fisher Sci	2320
Y-27632 Dihydrochloride	Abmole Bioscience	M1817

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Title of Article: Three-Dimensional (3D) organoids from patient tumor specimens and patient-derived xenografts of bone metastatic prostate cancer and its applications

Author(s): Sanghee Lee, Danielle Burner, Theresa Mendoza, Michelle Muldong, Catalina Arreola, Christina Wu, Nicholas Cacalano, Anna Kulidjian, Christopher Kane, Christina A.M. Jamieson

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July 5th, 2019

Dr. Ronald Myers
Senior Science Editor
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Re: Revisions required for your JoVE submission JoVE60367

Response to Editorial Comments

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
   We proofread our manuscript to avoid spelling or grammar issues.

2. Please provide the emails and affiliations for each author in the written manuscript.
   Our revised manuscript includes the emails and affiliations for each author.

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3. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to…"
Revised as following (Line 529).

Here, we present a protocol to establish, image and process cultured 3D organoid with detailed tips for successful completion of the desired procedures.

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Revised accordingly as following in a revised manuscript.
line 139 and 162, ~~QuadroMACS™~~
line 143, ~~Accumax~~ Cell Dissociation Solution ~~(Stem Cell Technologies)~~
line 267, ~~Keyence~~ microscope
line 277 and 355, ~~BZ-X800~~

Revision above was also reflected to the Table for Materials and Reagents.

5. Please include an ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.
Revised as following (Line 106); This study was carried out in strict accordance with the recommendations in the Guide for the University of California San Diego (UCSD) Institutional Review Board (IRB). IRB#090401 Approval was received from the UCSD institutional review board (IRB) to collect surgical specimens from patients for research purposes. An informed consent was obtained from each patient and a surgical specimen from fractured bone having prostate cancer metastasis was harvested and processed. All animal protocols were performed under a University of California San Diego (UCSD) animal welfare and Institutional Animal Care and Use Committee (IACUC) approved protocol #S10298. Harvest and processed patient tumor cells were intra-femorally injected in 6 to 8 week old male *Rag2*$^{-/-}$;$\gamma_c^{-/-}$ mice as previously described. Tumor volume was determined using an in vivo bioluminescence imaging system and caliper measurement. Upon tumor growth up to 2.0 cm (the maximal allowable size approved by IACUC), the tumor was harvested for 3D organoids establishment.

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6. Please convert centrifuge speeds to centrifugal force (x g) intead of revolutions per minutes (rpm).
Revised accordingly (line 135, line 188, line 322, line 328, line 333, line 336, line 315).

7. The protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step.
Our original version of protocol contained only 2-3 actions per step unless an additional note were written to share tips specifically applicable to each step.
In our revised version, some of the previously written notes were moved to Discussion to fulfil both request #7 and #14 shown as following.

Removed from line 134 and the edited sentences added to line 444;
After a one-time process of filtering the tumor tissue suspension, the left over tissue should be processed again by taking the filtered through supernatant from the bottom of the 50 ml conical tube back and pushed back through the 70 μm cell strainer with the tumor tissue for a repeated suspension.

Removed from line 195 and added to line 449;
In general, the establishment of 3D organoids from patient primary tumor tissue is less successful than the establishment of 3D organoids from PDX tumor tissue. Therefore, it is recommended to have a higher seeding density (up to four times higher) of cells for the establishment of 3D organoids from patient primary tumor tissues than from PDX tumors.

Removed from line 207 and edited sentences added to line 485;
For all three different methods, this protocol recommends using the lower volume of media and the higher volume of Matrigel for the resuspension process because the domes will last longer in culture. A dome is formed with a 1:4 ratio of media and Matrigel. Of note, this method increases the formation of bubbles while pipetting the cell suspension up and down since the added volume of media does not circumvent the viscosity of Matrigel. Therefore, it is recommended to set up 1.5 ml tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In doing so, the formation of bubbles in one tube for one dome will not serially affect the other domes.

Liquid status of commercially available Matrigel is often variable. Therefore, new batches of Matrigel need to be tested for the doming

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process. If the Matrigel is more viscous than usual, then up to additional 10 μl of adDMEM complete media (details written in protocol 1) can be added to dilute the mixture of cell pellet and matrigel.

Removed from line 205 and edited sentences added to line 437,
To minimize bubble formation during the pipetting process, it is recommended to pipette up a lower volume than the actual volume of cells, media and Matrigel mixture. For example, to dome in 24 well plate, 40 μl of Matrigel and up to 10 μl of adDMEM complete media is mixed with the cell pellet. In this case, the pipette can be set to handle a volume of 40 μl instead of 50 μl while pipetting up and down in order to minimize bubble formation. If the duration of culture condition is previously optimized and known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue of the Matrigel. For this second method, cell pellet resuspension for several wells can be prepared in one tube to minimize cell loss and variability.

8. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
   Revised accordingly.

9. The preparation of the material steps can be removed or please refer to the Table of Materials.
   The preparation of the material steps was removed from line 127, 260 and line 319.

10. What is the age/gender/strain of the mouse used?
    Revised as following in line 113;
    Harvested and processed patient tumor cells were intra-femorally injected in 6 to 8 week old male *Rag2*$^{-/-}$;$\gamma_c^{-/-}$ mice as previously described.

11. 1.2.d: The units of the cell strainer are wrong.
    Revised accordingly in line 132, line 133 and line 447.

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12. 1.2.4: Wash with what volume of media.
Revised accordingly.

In line 166, following statements were added;
As shown in Table 3, we aim to provide a protocol applicable to different culture conditions. Volume of media for washing step should be matched with volume of media suggested in Table 3. For example, for a 24 well plate culture condition, the volume for the wash should be 500 µl.

13. Please specify the volumes of all washes and resuspensions.
Revised accordingly.

14. Please discuss limitations and critical steps on the protocol in the discussion.
Revised accordingly as following;

After a one-time process of filtering the tumor tissue suspension, the left over tissue should be processed again by taking the filtered through supernatant from the bottom of the 50 ml conical tube back and pushed back through the 70 µm cell strainer with the tumor tissue for a repeated suspension.

In general, the establishment of 3D organoids from patient primary tumor tissue is less successful than the establishment of 3D organoids from PDX tumor tissue. Therefore, it is recommended to have a higher seeding density (up to four times higher) of cells for the establishment of 3D organoids from patient primary tumor tissues than from PDX tumors.

For all three different methods, this protocol recommends using the lower volume of media and the higher volume of Matrigel for the resuspension process because the domes will last longer in culture. A dome is formed with a 1:4 ratio of media and Matrigel. Of note, this method increases the formation of bubbles while higher chance pipetting the cell suspension up and down since the added volume of media does not circumvent the viscosity of Matrigel.
Therefore, it is recommended to set up 1.5 ml
tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In doing so, the formation of bubbles in one tube for one
dome will not serially affect the other domes. If the duration of the culture condition has been previously optimized and is known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue of Matrigel. Cell pellet resuspension for several wells can be prepared in one tube, utilizing a higher volume of media to desired volume of Matrigel to minimize cell loss and variability.

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Liquid status of commercially available matrigel is often variable. Therefore, new batches of matrigel need to be tested for the doming process. If the matrigel is more viscous than usual, then up to additional 10 μl of adDMEM complete media (details written in protocol 1) can be added to dilute the mixture of cell pellet and matrigel.

15. Please do not abbreviate journal titles in the References.
Revised accordingly.

16. Please provide scale bars for all microscope images.
Revised accordingly.

17. Figure 3: Please use the SI abbreviation for minutes (min).
Revised accordingly.

18. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or xlsx file.
Revised accordingly.

19. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or docx. file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
None of figures in our manuscript has previously been published. Tables were obtained from previous publication as written in our original and revised manuscript and shared with readers for convenience. 'Copyright Permission Letter' is submitted for Tables. For table legends for Table 1, 2 and 3, a following statement was added in a revised manuscript.

Response to Reviewer #1' Comments

1. One question is raised by the fact that it is not clear why this protocol should be applied only to bone metastatic prostate tumors since it looks like just a good example of trying to culture organoids from limited amount of starting material. I would suggest revising the title to increase the applicability of the methods to different types of metastatic sites (specifying the amount of material used as a starting). Otherwise why the protocol is applicable only to bone? Authors should explain that in details.
Our manuscript is submitted to JoVE Methods Collection, which aims to introduce several manuscripts to describe the establishment of 3D orgsion in our revised manuscript line 497;
Culture media for 3D organoids introduced in our manuscript

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is specifically for prostate-derived cells. However, other details described in our protocol are applicable to different types of tumor tissues and metastatic sites.

2. Author should clarify why FBS is needed in their culture since it will allow the growth of non-tumor cells (example fibroblasts). How the author eliminates non-tumor cells from the culture?
   Our group recently determined that serum supplementation can help support 3D organoid growth. For 3D organoid cultures from tumor specimens, we do not aim to selectively culture a pure population of tumor cells but rather to support tumor growth with the patient-derived microenvironment cells as well. However, as previously published by J Drost et al, culture media used for our protocol is most effective for human prostate-derived epithelial tumor cells. In parallel, our goal for the establishment of 3D organoids from bone metastatic prostate cancer is to provide culture conditions for heterogeneous tumor cells to be able to retain their properties as closely as possible to their *in vivo* condition. *In vivo*, tumor cells require interaction with non-tumor cells of the microenvironment.

   The statement regarding FBS supplementation was revised (line 451) to the following:
   In other words, the culture conditions described here which were adapted from Drost et al and modified to have FBS serum supplementation have been shown to be optimal for the cultures of patient prostate cancer bone metastasis-derived cells so that they can survive, grow and also retain their heterogeneity (manuscript in prepration).

3. How the authors check that they have in culture is really tumor from a bone metastasis? And no other cell types? Any test on the tumors to include in the protocols?
   As written above in response to Reviewer #1's comment 2, there can be other cell types. During culture, we do not aim to selectively culture pure population of tumor cells for organoids growth. However, after termination of experiment, we can perform IFC to characterize heterogeneous population of cells using different cell markers.

4. Page 2 line 101 it looks like the protocol is just for organoids derived from PDX since a Mouse Cell Depletion Kit is mentioned. Please specify that this protocol is for tumor coming from mouse at the beginning of the paragraph.
   Revised accordingly in line 123,
   for tumor derived from a xenograft mouse model

5. Is the protocol explained from line 189 the one to be used for Imaging? Please specify if the two paragraphs are connected.
   Each protocol to be used for each figure was labelled with 'Figure #' in our original and revised manuscript. Protocols which will be video filmed were yellow highlighted in response to a request by JoVE

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editorial team.

6. Literature references for prostate organoids establishment and applications are missing as Vela et al, 2014 and Puca et al., 2018.
   Revised accordingly.

7. Page 4 line 186 please remove the word "either"
   Revised accordingly.

8. Authors should clarify what they are referring to when they talk about "beads"
   Following sentence was added to our revised manuscript (line 259); NOTE: This protocol is named as floating beads since the mixture of Matrigel, media and organoids looks like a bead.

Response to Reviewer #2' Comments

1. When plating the organoids Line 157:
   "(NOTE: For this process, it is recommended to set up 1.5 ml tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In this way, cell loss is minimized and cell density between domes is uniform when plating.)"
   This rather seems to induce more variability akin to making a separate master mix for each qPCR well. The viscosity of the Matrigel is the main factor in creating seeding variability. However, by diluting the Matrigel to 70% with medium this can be circumvented.
   a. **We prefer to use the lower volume of media and higher Matrigel for resuspension process because the domes last longer in culture. A dome formed with ratio less than 1:4 media:Matrigel shows slower de-polymerization and degradation in culture for long term cultures.**

   b. We recommend preparing the mix for each dome individually rather than from a common stock. This is to avoid bubbles that would arise from successive resuspension if a master mix of Matrigel + cells were to be used. Unlike qPCR, an exponential amplification reaction, this is a more accurate way to prepare the cells + Matrigel which is highly viscous.

   Original statement was removed from line 157 and then revised and added to Discussion in response to Editorial request.
   For all three different methods, this protocol recommends using the lower volume of media and the higher volume of Matrigel for the resuspension process because the domes will last longer in culture. A dome is formed with a 1:4 ratio of media and Matrigel. Of note, this method increases the formation of bubbles while pipetting the cell suspension up and down since the added volume of media does not circumvent the viscosity of Matrigel.
   Therefore, it is recommended to set up 1.5 ml tubes such that there is one cell pellet per each dome instead of having one cell pellet for several domes in one 1.5 ml tube. In doing so, the formation of bubbles in one tube for one dome will not serially affect

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the other domes. If the duration of the culture condition has been previously optimized and is known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue of Matrigel. Cell pellet resuspension for several wells can be prepared in one tube, utilizing a higher volume of media to desired volume of Matrigel to minimize cell loss and variability.

To minimize bubble formation during the pipetting process, it is recommended to set up the pipette up a lower volume than the actual volume of the cells, media and Matrigel mixture For example, for a dome within 24 well plates, 40 µl of Matrigel and up to 10 µl of adDMEM complete media are mixed with cell pellet. In this case, the pipette can be set to handle a volume of 40 µl instead of 50 µl while pipetting up and down in order to minimize bubble formation. If the duration of culture of culture condition is previously optimized and known to be short (up to a month), Matrigel can be diluted more to circumvent a viscosity issue the Matrigel. For this second method, cell pellet resuspension for several wells can be prepared in one tube to minimize cell loss and variability.

2. In the image analysis is it possible to see the difference between dying cells that blow up with big vacuoles and cysts?
We have not tried to determine dying cells in the image. We have optimized a condition for IFC staining with specific markers.

3. This is the biggest point of critique.
They show different ways to plate Matrigel discs and domes, attached or unattached, which is fine. However, the discussion of when you would use each of these techniques and the pros and cons of each is largely absent. A couple of examples are given in the discussion but this needs to be much more complete as this comprises a large proportion of the protocol presented. For instance, there are a couple different ways to plate floating domes, why use one or the other? As one is clearly much easier than the other.

Following sentences were added to line 477;
In addition, both types of floating domes can be easily transferred from one well to the another, making them suitable for co-culture with adherent cells after setting up 3D organoids cultures and the adherent cells in the separate wells. Attached dome and floating dome released from being attached have shown to retain organoids for up to 10 weeks. Interestingly, a floating dome from floating beads method has shown to retain organoids for up to 4 months. Floating beads method introduced in our protocol involves more steps and skills but can be considered for a long-term culture. In parallel, despite their use to establish 3D organoids from different tumors and metastasis, the role of doming method and Matrigel shape on organoids formation have not been deeply determined. In that sense, floating beads method can be considered

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for use when the two other methods have been unsuccessful.

4. Line 7: its should be their
   We thank for your suggestion. In our revised version of manuscript, we revised a title so that this is not applicable to a current version of title.

5. Line 173: typo (Issue instead of Tissue)
   Revised accordingly
6. Line 265: Some comments from other authors during manuscript preparation phase remain.
   Revised accordingly

We truly appreciate the careful consideration to give us an opportunity to publish or manuscript JoVE60307 in JoVE Methods Collection. If you have any questions and further request, please let us know.

Sincerely,

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July 5th, 2019

Dr. Vineeta Bajaj
Review Editor
JoVE, Peer Reviewed Scientific Video Journal
617.674.1888

Re: Revisions required for your JoVE submission JoVE60367 – Copyright permission Letter

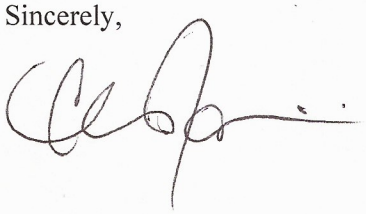
Dear Dr. Bajaj,

Thank you very much for considering our manuscript for publication in JoVE Methods Collection.

In this letter, we would like to have your permission to reuse two tables (Table 1 and Table 2 labelled in our manuscript JoVE 60367 from the previous publication entitled "Organoid culture systems for prostate epithelial and cancer tissue" by J Drost, W Karthaus, D Gao, E Driehuis, C L Sawyers, Y Chen and H Clevers. In addition, Table 3 in our manuscript JoVE60307 was modified from a table previously published by J Drost et al.

Thank you for the opportunity to share our work with the readers of the JoVE, Peer Reviewed Scientific Video Journal.

Sincerely,



Christina A.M. Jamieson, Ph.D