Video Article

# Human Neuroendocrine Tumor Cell Lines as a Three-Dimensional Model for the Study of Human Neuroendocrine Tumor Therapy

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URL: https://www.jove.com/video/4218
DOI: doi:10.3791/4218

Keywords: Medicine, Issue 66, Medicine, Neuroscience, Cell Culture, Tissue Engineering, 3D model, multicellular spheroids, therapeutic drugs, neuroendocrine tumor cell lines, agarose overlay platform, paraffin embedding

Date Published: 8/14/2012

Citation: Wong, C., Vosburgh, E., Levine, A.J., Cong, L., Xu, E.Y. Human Neuroendocrine Tumor Cell Lines as a Three-Dimensional Model for the Study of Human Neuroendocrine Tumor Therapy. *J. Vis. Exp.* (66), e4218, doi:10.3791/4218 (2012).

## Abstract

Neuroendocrine tumors (NETs) are rare tumors, with an incidence of two per 100,000 individuals per year, and they account for 0.5% of all human malignancies.[1] Other than surgery for the minority of patients who present with localized disease, there is little or no survival benefit of systemic therapy. Therefore, there is a great need to better understand the biology of NETs, and in particular define new therapeutic targets for patients with nonresectable or metastatic neuroendocrine tumors. 3D cell culture is becoming a popular method for drug screening due to its relevance in modeling the *in vivo* tumor tissue organization and microenvironment.[2,3] The 3D multicellular spheroids could provide valuable information in a more timely and less expensive manner than directly proceeding from 2D cell culture experiments to animal (murine) models.

To facilitate the discovery of new therapeutics for NET patients, we have developed an *in vitro* 3D multicellular spheroids model using the human NET cell lines. The NET cells are plated in a non-adhesive agarose-coated 24-well plate and incubated under physiological conditions (5% $CO_2$, 37 °C) with a very slow agitation for 16-24 hr after plating. The cells form multicellular spheroids starting on the 3$^{rd}$ or 4$^{th}$ day. The spheroids become more spherical by the 6$^{th}$ day, at which point the drug treatments are initiated. The efficacy of the drug treatments on the NET spheroids is monitored based on the morphology, shape and size of the spheroids with a phase-contrast light microscope. The size of the spheroids is estimated automatically using a custom-developed MATLAB program based on an active contour algorithm. Further, we demonstrate a simple method to process the HistoGel embedding on these 3D spheroids, allowing the use of standard histological and immunohistochemical techniques.

This is the first report on generating 3D spheroids using NET cell lines to examine the effect of therapeutic drugs. We have also performed histology on these 3D spheroids, and displayed an example of a single drug's effect on growth and proliferation of the NET spheroids. Our results support that the NET spheroids are valuable for further studies of NET biology and drug development.

## Video Link

The video component of this article can be found at https://www.jove.com/video/4218/

## Protocol

### 1. Preparation of 1% Agarose-coated 24-well Plate

1. Add 1 g agarose (RPI, A20090-500) to 100 ml deionized water in a 250-500 ml bottle and autoclave to sterilize the agarose (121 °C, 15 psi, 15 min in an autoclave machine). Transfer the bottle with sterilized agarose into a 70 °C water bath. The agarose is ready to be poured when it's cooled to 70 °C, which takes about one hour. Make sure to keep the agarose sterile at all times.
2. Obtain the 24-well flat-bottom plates (Falcon, 353047); any kind of 24-well flat-bottom plates would work if the plates can be imaged under a phase-contrast microscope.
3. Dispense 200 µl of agarose to each well with an Eppendorf repeater pipette in a biosafety cabinet and swirl the agarose around the well to make it cover the well evenly. 200 µl of agarose is appropriate to form a concave surface so that spheroids can form the consistent spherical shapes. Less than 200 µl agarose may not cover the well entirely, but more than 200 µl agarose may not form the concave surface and the spheroids grown on it may not form spherical shapes.
4. The agarose-coated 24-well plates are ready to be used in about 10 min. (Optional) Add 100 µl of medium to equilibrate the agarose with culture medium prior to seeding cells.
5. Alternatively, after the agarose is fully solidified, add 100 µl growth medium to each well and seal the plates with the sterile sealing tape (Nunc, 236366). These plates can be stored at 4 °C for up to one week.

## 2. Preparation of Single Cell Suspensions

1. All the experiments are done using the standard aseptic techniques in a biosafety cabinet and all the cultures are grown in a 37 °C humidified incubator with 5% $CO_2$ in air, unless specified.
2. Human pancreatic NET BON-1 cells are maintained in DMEM/F12 (Invitrogen, 10565) with 10% fetal bovine serum (FBS, Invitrogen, 16000-044) medium in a 100 mm x 20 mm sterile tissue culture dish (Corning, 430167).
3. When monolayer BON-1 cells reach 70-80% confluency, medium is removed. The cells are washed with PBS twice, and dislodged from the dish by adding 1 ml TrypLE (Invitrogen, 12604) with 5 min incubation at 37 °C. TrypLE is similar to trypsin but is stable at 15 - 30 °C as well as at 4 °C. Check under microscope to make sure all cells are detached from the dish.
4. Add 9 ml growth medium to the trypsinized cells and use a pipette to pipette cells up and down a few times to ensure an even single cell suspension.
5. Use 70-µm cell strainer (Fisher, 22363548) to filter the cells.
6. Collect cells and perform a cell count using Guava PCA-96 Base System (Millipore).
7. Prepare a suspension of 5,000 cells per ml in phenol-free DMEM/F12 with 10% FBS growth medium.

## 3. Spheroid Culture

1. Overlay 200 µl of the single cell suspensions to the agarose-coated 24-well plate and seal the 24-well plates with the sterile sealing tape to prevent evaporation.

Note: --The number of cells to be plated should be determined empirically based on the size of the spheroids at day 6; 300-400 µm diameter of the spheroids would be the ideal size for starting the drug treatment; 1,000 BON-1 and H727 cells are plated.

-- We don't recommend starting drug treatments after day 6 because the viability of the 3D cells starts to drop at about day 10 (data not shown, manuscript in preparation).

2. Place the above agarose-coated 24-well plates with BON-1 cells on an orbital shaker at a very slow agitation of less than 40 rpm overnight in a 37 °C humidified incubator with 5% $CO_2$ in air. Then move these plates to a stable platform to keep the cultures growing.
3. Add 200 µl growth medium to each well of the above plates every 3 days. Do not disturb the spheroids. Try to add medium through the wall of each well by touching the pipette tip to the side of the well and letting the medium flow down into the well.
4. Monitor the spheroid formation in the 24-well plates under a microscope.

## 4. Drug Treatment

1. When the spheroids reach around 300-400 µm in diameter at day 6, the 3D spheroids are treated with drugs. This is denoted as day 0 for drug treatment.
2. At day 6, each well of the plate contains around 400 µl of medium. To make sure the drug treatments are at 1x final concentration in each well, 3x serial dilutions of each dose of treatments are made from the drug stock in 5 ml growth medium in a 15 ml conical tube, which is enough for 8 replicates.
3. The spheroids at each row of the 24-well plate are categorized as vehicle, dose 1, 2, 3, 4 and 5. There are 4 replicates for each dose of treatments on one 24-well plate (see **Table 1**). We usually treat 3D spheroids at least in 2 24-well plates, which makes at least 8 replicates for each dose of treatments.
4. Spin down the 24-well plate at 800 rpm for 5 min at 4 °C to make sure that all the condensations on the sealing tape go into the well. Due to the floating feature of the 3D spheroids, the medium in each well is not removed to avoid the disturbance of the spheroids.
5. Carefully add 200 µl of the above-made treatments of each dose into the appropriate wells along the wall of each well.
6. Seal the 24-well plates with the sealing tape and incubate the cultures in the 37 °C humidified incubator with 5% $CO_2$ in air.
7. If necessary, retreat the spheroids at each well with the respective dose of treatments after 72-96 hr.

## 5. Monitor 3D Spheroid Cultures

1. At each chosen time point after drug treatments (0, 24, 48, 72 hr), the spheroids at each well of the 24-well plate are imaged with a Zeiss Axiovert 200/M-based phase-contrast microscope using a 5x objective.

Note: Images can be taken on any light microscope with a calibrated scaling between µm and pixel. A 5x objective is ideal because the spheroids quickly outgrow the imaging field with a 10x objective.

2. Spheroid analysis can be performed manually using Zeiss AxioVision 4.8.2 Software with the raw images.
3. Alternatively, a custom-developed MATLAB program is used to estimate the size of the spheroids automatically/semi-automatically. The automatic program implements the active contour algorithm[4] to accurately locate contour of the spheroid in a given image and measure its major (L) and minor (W) axes automatically. A semi-automatic version of the same algorithm allows users to help initiate the program when strong artifact is present. All the images need to be exported as TIFF or JPEG file formats from the raw images to be read by the program. The volume of the spheroid is estimated as $0.5 \times L \times W^2$.

## 6. HistoGel-embedding of the NET 3D Spheroids

1. 10-24 spheroids are collected from the 24-well plates, washed in PBS twice, fixed in 10% formalin for 2 hr, washed in 50% and 70% ethanol for 15 min twice, respectively, and are ready for HistoGel embedding. Alternatively, they can be kept in 70% ethanol at 4 °C for several months.
2. A tube of cold HistoGel (Thermo Scientific, HG-4000-012) is put in a beaker filled with water. The beaker is heated in a microwave for 1 min or until the gel is completely liquefied, then kept in the same water-filled beaker at all times.
3. The spheroids in 70% ethanol from above are transferred into a biopsy cryomold (Tissue-Tek, Sakura Finetek, 4565) using a toothpick. Make sure all the spheroids are transferred. Let the spheroids sit for a minute to sink down to the bottom of the biopsy cryomold. Try to get rid of the liquids in the biopsy cryomold with Kimwipes and meanwhile, very gently push the spheroids to the center at the bottom of the biopsy cryomold using the disposable plastic Pasteur pipette. This step may be easier to be done under a dissecting microscope.
4. A thin layer of warmed HistoGel is added to the biopsy cryomold to stabilize the spheroids at the bottom of the biopsy cryomold; meanwhile, the toothpick is used to keep the spheroids at the center at the bottom of the biopsy cryomold very gently. Don't add too much HistoGel but just enough for stabilizing the spheroids at the bottom of the biopsy cryomold.
5. Let the spheroids/HistoGel sit for 1-2 min at room temperature or till the HistoGel is solidified. Then fill the rest of the biopsy cryomold with the warmed HistoGel.
6. Allow it to solidify at room temperature for about 10 min.
7. Just prior to popping out the spheroids/HistoGel block from the biopsy cryomold, leave it at 4 °C for 1 min, which helps popping out the intact spheroids/HistoGel block. The spheroids/HistoGel block is then wrapped in a piece of Bio-wraps (Surgipath, 01090) and put into a tissue and biopsy cassette (Fisher Scientific, 15-182-701D). The tissue and biopsy cassette is stored in a container with 70% ethanol. Then the routine tissue processing procedures for paraffin embedding are followed.[5]
8. The paraffin block can be sectioned into 3-µm thick layer slides. The slides can be stained with hematoxylin and eosin to determine histological changes and can also be used for immunohistochemical staining.

## 7. Representative Results

Human pancreatic neuroendocrine tumor cell lines BON-1 (provided to Verto Institute by Kjell Oberg, Uppsala University, Sweden), QGP-1 (Japanese Health Sciences Foundation, Osaka, Japan), and human lung neuroendocrine tumor cell line H727 (ATCC) were grown on an agarose-coated 24-well plate. As seen in **Figure 1**, the three cell lines show different morphology. BON-1 and H727 cells formed 3D spheroids. Under the microscope, H727 cells showed tighter and more compact spheroids than BON-1 cells. We hypothesize that H727 cells have an increased capacity for cell-cell adhesion and form tighter junctions as a result. QGP-1 cells show large grape-like porous masses, which are not regarded as spheroids. Due to the rising interest of pancreatic neuroendocrine tumor research in recent years, BON-1 cells are used for the rest of the figures. After seeding BON-1 cells on the agarose-coated 24-well plate, multicellular spheroids formation typically occurs by the 3$^{rd}$ or 4$^{th}$ day and the spheroids become rounder by the 6$^{th}$ day. Due to the nutrient and oxygen limitation, cells in the dense core of the spheroids start to die around day 10 and by day 17, the spheroids begin to disintegrate due to the large size or in some cases the ejection of the necrotic core. The spheroids typically can grow up to 1,000 µm in diameter. **Figure 2** shows the sections of the formation, growth and disintegration of BON-1 3D spheroids.

Drug treatments were started when spheroids were around 300-400 µm and cells retained high viability (manuscript in preparation). Day 6 3D spheroids were chosen as the start point for drug treatments. It has been reported that histone deacetylase inhibitors showed antiproliferative and proapoptotic effects on the NET cells.[6] We chose the histone-deacetylase inhibitor -Trichostatin A (TSA) as an example to illustrate the effect of drug treatments on 3D NET spheroids. **Figure 3A** shows the morphology of the 3D spheroids upon the treatment of TSA. **Figure 3B** shows the volume of the 3D spheroids upon TSA (Sigma, T8552) treatment. The size of each individual spheroid was estimated automatically by a custom-developed MATLAB computer program.[4] A manual drawing step was added to help acquire the spheroids, which were close to the edge in a given image. At least 8 spheroids were measured per dose of TSA treatments at each time point. The volume of each 3D spheroid was calculated as 0.5 x Length x Width$^2$. As seen in **Figure 3A and 3B**, relative to vehicle, all doses of TSA shown in the figures demonstrated some degree of growth inhibition of BON-1 3D spheroids. Among them, the concentrations of 125 nM and 250 nM of TSA strongly suppressed the growth of 3D spheroids and led to cell death at 96-hr time point.

To understand the histology of the NET 3D spheroids, an H & E and immunohistochemical (IHC) staining were performed on 3D spheroids that had been cultured for 17 days. **Figure 4A** illustrates a method to process 3D spheroids for HistoGel embedding, which is the key step for paraffin embedding of 3D spheroids. **Figure 4B** shows the H & E staining, the immunohistochemical staining of the cleaved caspase-3 (a marker for apoptotic cells) and Ki-67 (a marker for proliferating cells) antibodies on 3D spheroids, which had been cultured for 17 days. The cells within the core of the 3D spheroids are mostly necrotic/apoptotic and the outer layer cells are actively proliferating.

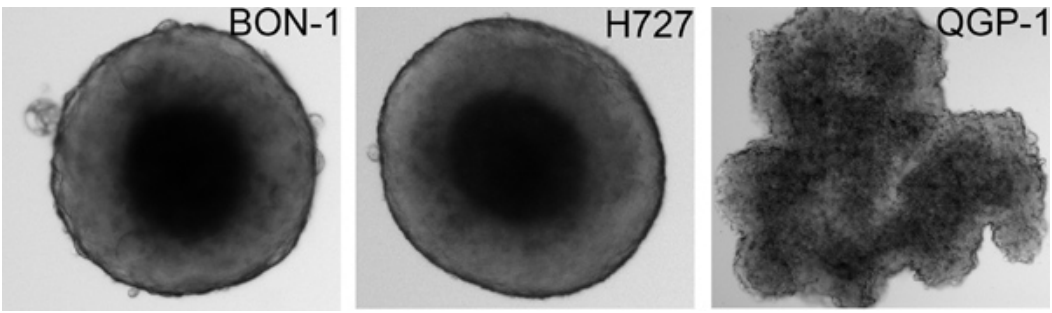


**Figure 1. The Morphology of NET 3D Spheroids.** The 3D spheroids from three human neuroendocrine tumor cell lines are formed on agarose-coated 24-well plates. 1,000 BON-1, H727 and QGP-1 cells were seeded onto agarose-coated 24-well plates and grown in a normal

physiological condition as detailed in the protocol. These are the images of 3D spheroids for BON-1 and H727 or cell aggregates for QGP-1, which were cultured for 10 days.

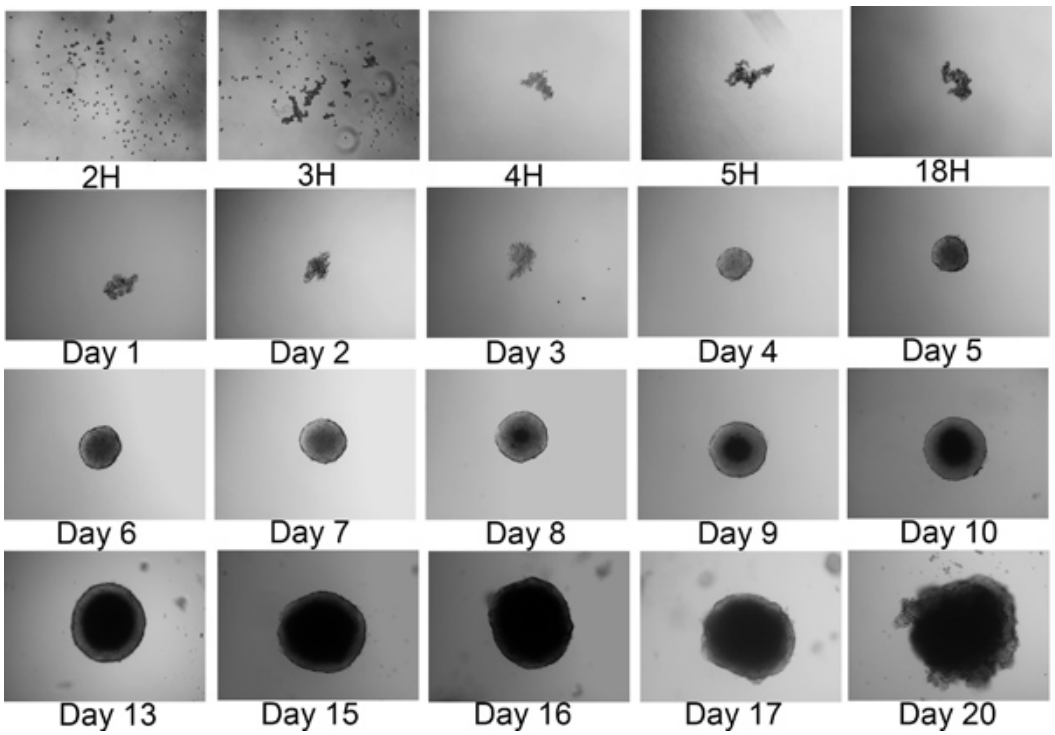


**Figure 2. The Growth Tracking of BON-1 3D Spheroids.** The growth tracking of BON-1 3D spheroids: formation, growth and disintegration of 3D spheroids. As detailed in the protocol, 1,000 BON-1 cells are plated onto an agarose-coated 24-well plate. Cells are grown in a standard DMEM/F12 medium with 10% FBS on an orbital shaker in a physiological condition overnight, then moved on a stable platform and grown in the same condition. The growth tracking of the 3D cells were followed by imaging cells every hour for the first 5 hr after plating, and then every 1-3 days with a Zeiss Axiovert 200/M-based phase-contrast microscope using a 5x objective. The cells aggregate into masses first, then form multicellular aggregates, small spheroids, bigger spheroids, and eventually the spheroids disintegrate.

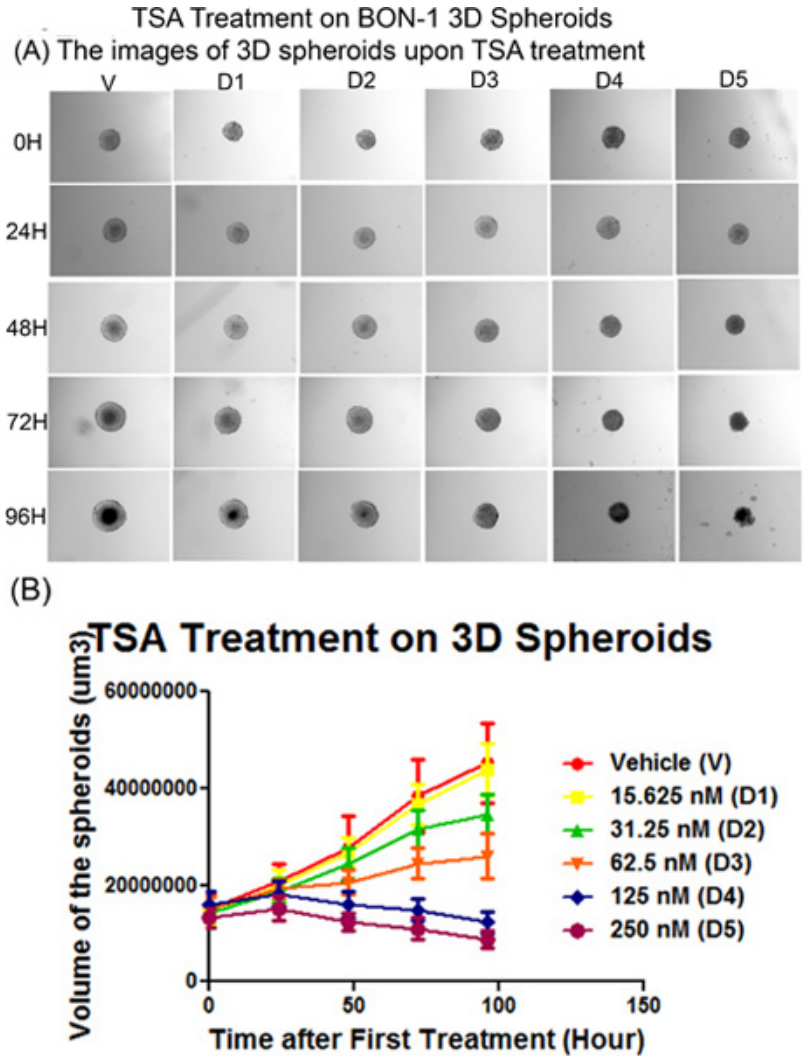


**Figure 3. TSA Treatment on BON-1 3D Spheroids.** (A) Images of 3D spheroids upon TSA treatment. Treatment group: V - Vehicle (0.0025% DMSO); D1- dose 1 (15.625 nM TSA); D2- dose 2 (31.25 nM TSA); D3: dose 3 (62.5 nM TSA); D4: dose 4 (125 nM TSA); D5: dose 5 (250 nM TSA). Time of treatment: 0 H - Time point that treatments started on day 6 3D spheroids; 24 H, 48 H, 72 H and 96 H are the time points after the treatments started. 3D spheroids were imaged at each time point as before. (B) Growth of the 3D spheroids upon TSA treatment. The major (L) and minor (W) axes of the 3D spheroids in (A) were estimated automatically by Matlab program at each time point of treatments (0, 24, 48, 72 and 96 hr). The volume of the 3D spheroids was estimated as $0.5 \times L \times W^2$. The volume of the spheroid in the graph was the average of 8 replicates and the error bar was calculated base on the 8 replicates.

| | Vehicle (1) | Dose 1 (2) | Dose 2 (3) | Dose 3 (4) | Dose 4 (5) | Dose 5 (6) |
|---|---|---|---|---|---|---|
| Replicate 1 (Row A) | spheroids | spheroids | spheroids | spheroids | spheroids | spheroids |
| Replicate 2 (Row B) | spheroids | spheroids | spheroids | spheroids | spheroids | spheroids |
| Replicate 3 (Row C) | spheroids | spheroids | spheroids | spheroids | spheroids | spheroids |
| Replicate 4 (Row D) | spheroids | spheroids | spheroids | spheroids | spheroids | spheroids |

**Table 1.** Layout of the drug treatments on a 24-well plate.

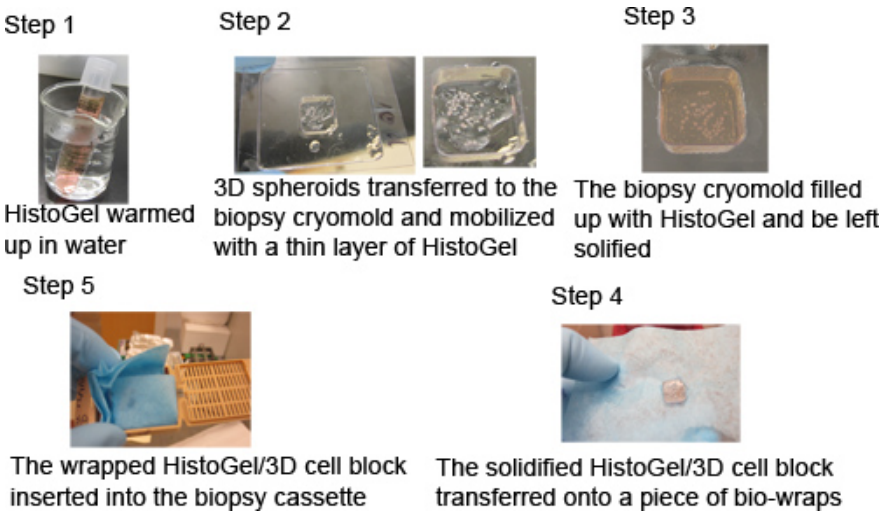


**Figure 4A. Illustration of a Method to Process 3D Spheroids for HistoGel Embedding.** A method to process 3D spheroids for HistoGel embedding and immunohistochemical staining of the 3D spheroids (A) An overview of a method to process 3D spheroids for HistoGel embedding. 3D spheroids were encapsulated in a HistoGel to be at the same level in the biopsy cryomold, then the HistoGel/spheroids block was wrapped in a blue Bio-wraps and transferred into a yellow biopsy cassette for paraffin embedding.

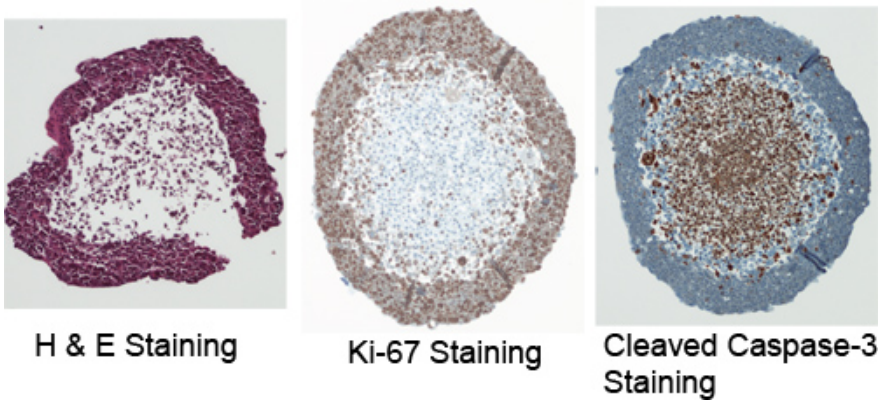


**Figure 4B. H & E Staining and Immunohistochemical Staining of day-17 3D Spheroids.** (B) H & E staining, Immunohistochemical staining of Ki-67 and Cleaved Caspase-3 of the 17-day 3D spheroids. The paraffin-embedded sectioned slides were deparaffinized and antigen retrieval was performed using CCI (Cell Conditioning Solution, Verana Medical System, #711-065-152). Rabbit polyclonal Ki-67 antibody (Abcam. #Ab833) and Cleaved Caspase 3 antibody (Cell Signaling Technology, #9661) were applied at a concentration of 1:75 and 1:200, respectively, for 1 hr at room temperature. The anti-rabbit secondary antibody (Jackson Immunolab, #711-065-152) was applied at 1:500 for 1 hr at room temperature, followed by chromogenic detection kit DABMap (Ventana Medical Systems, #760-124). Slides were counterstained with Hematoxylin and dehydrated and cleared before coverslipping from Xylene.

## Discussion

We have demonstrated a simple, reliable, and reproducible method for forming 3D multicellular spheroid from human neuroendocrine tumor cells using agarose-coated 24-well plates. The success of this technique is the ability to obtain a single spheroid with homogenous size and uniform shape by day 6 starting from a fixed number of cells. The key step is the slow agitation of the plate with NET cells for the first 16-24 hr after plating. This method can also apply to common human breast cancer cell line MCF-7, human colon adenocarcinoma cell line HT-29, human non-small cell lung carcinoma cell line H1299 and human embryonic kidney cell line HEK-293. They can all form uniform and compact 3D spheroids for therapeutic drug screen (Data not shown). Although several methods are reported on generating 3D spheroids,[7-10] the method reported here does not require specialized plates or reagents and is thus simple and cost-effective. Our unpublished data demonstrate that NET 3D spheroids grown on agarose mimic the *in vivo* NET xenograft tumor geometrically and molecularly, when compared to monolayer 2D cells. This is similar to what has been reported for the 3D spheroids made from on other human cancer cell lines.[11,12] The limitation of the 3D cell culture model is that it cannot completely replace testing the *in vivo* efficacy of any drugs or therapeutic treatments because the 3D spheroids are like avascular tumors, which is different from the *in vivo* tumor in this aspect. Nonetheless, this method will help bridge the gap and assessments of NET therapeutic drugs from *in vitro* to *in vivo* in some aspects.

Immunofluorescence staining on multicellular spheroids is very difficult to perform due to the large size and geometry of the floating 3D spheroids and the limitation of the confocal microscope. Here we illustrate a method to process HistoGel embedding for paraffin embedding, permitting immunohistochemical staining of the serial sectioned slides of the 3D spheroids. Besides, our custom-developed MATLAB program helps accurately and reproducibly estimate the size of the spheroids, monitor the drug efficacy efficiently, and could be incorporated into a high-throughput screen in the development of drugs for NET patients.

## Disclosures

No conflicts of interest declared.

## Acknowledgments

We sincerely thank Dr. Wenjin Chen for her help with the MATLAB program and the Cancer Institute of New Jersey histopathology facility and microscopy facility. We also thank the reviewers for their comments and suggestions. This study is supported by the Raymond and Beverly Sackler Research Foundation.

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