Submission ID #: 61141
Scriptwriter Name: Anastasia Gomez
Project Page Link: https://www.jove.com/account/file-uploader?src=18648053

Title: Advanced 3D Liver Models for In vitro Genotoxicity Testing Following Long Term Nanomaterial Exposure

Authors and Affiliations:

Samantha V. Llewellyn, Gillian E. Conway, Ume-Kulsoom Shah, Stephen J. Evans, Gareth J.S. Jenkins, Martin J.D. Clift, Shareen H. Doak

In vitro Toxicology Group, Swansea University Medical School, Swansea, UK

Corresponding Authors:

Samantha V. Llewellyn and Professor Shareen H. Doak

Email Addresses for All Authors:

S.V.Llewellyn@swansea.ac.uk
gillian.conway@swansea.ac.uk
U.shah@swansea.ac.uk
S.J.Evans@swansea.ac.uk
G.J.S.Jenkins@swansea.ac.uk
M.J.D.Clift@swansea.ac.uk
S.H.Doak@swansea.ac.uk

Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **No**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Gillian E. Conway:** This 3D liver spheroid model protocol provides a relevant alternative in vitro testing system to more reliably assess the potential induction of fixed DNA damage following long-term exposure to nanomaterials, with an aim to minimize the need for testing in animals.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Gillian E. Conway:** The HepG2 spheroid model possesses the ability to remain viable and functional over a 14-day period as well as sustain a suitable level of proliferation to be able to support the testing of a range of biochemical and genotoxicity endpoints, including the micronucleus assay.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Samantha V. Llewellyn:** When attempting this protocol, take your time, do a few practice runs, and take extra care when seeding cells or changing media as it requires a delicate approach that can take a bit of getting used to.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~1.4. **Samantha V. Llewellyn:** Visual demonstration of this method makes it possible to show how the HepG2 spheroids are formed on the lid of a 96-well plate. Without a visual aid it is quite difficult to explain the logistics of setting up the hanging-drop cell culture.~~

~~1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.~~

Protocol

2. HepG2 Spheroid Preparation

2.1. Begin by adding 100 microliters of sterile, room temperature PBS to the wells of a 96-well culture plate **[1]**. Invert the lid of the plate and carefully pipette 20 microliter drops of the cell suspension into the center of each well groove of the lid. Use a multi-channel pipette, adding 2 to 4 drops at a time to ensure accuracy of placement **[2]**. *Videographer: This step is important!*

2.1.1. WIDE: Establishing shot of talent adding PBS to the plate.

2.1.2. Talent pipetting cells onto the lid of the plate, slowly and dropwise.

2.2. **Samantha V. Llewellyn:** Only seed 4 spheroids at a time and ensure the pipette tips are all aligned straight before beginning. Furthermore, the angle at which you hold the multi-channel can make a difference in the way the spheroid droplets are formed.

2.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

2.3. Make sure that the drops are centered within the grooves of the wells on the lid **[1]**, then gently flip the lid on top of the plate so that the drops hang over the wells with PBS **[2]**. Incubate the plate at 37 degrees Celsius and 5% carbon dioxide for 3 days prior to spheroid transfer onto agarose **[3]**. *Videographer: This step is important!*

2.3.1. Lid with the drops centered within the well grooves.

2.3.2. Talent gently flipping the lid. *Videographer: If possible, get an ECU of the plate lid with the drops hanging.*

2.3.3. Talent putting the plate in the incubator and closing the door.

2.4. After the incubation, remove the plate from the incubator and carefully lift the lid off the plate **[1]**. Discard the PBS, tap the plate to remove any residual liquid and allow the plates to airdry for 2 to 3 minutes **[2]**. *Videographer: This step is important!*

2.4.1. Talent removing the lid off of the plate.

2.4.2. Talent removing the PBS from the lid.

2.5. After melting the agarose, gently swirl it to remove any bubbles **[1]** and add 50 microliters into the base of each well. Leave the plate at room temperature for 2 minutes **[2]**, then add 100 microliters of pre-warmed DMEM on top of the solid agarose layer in each well **[3]**. *Videographer: This step is important!*

2.5.1. Talent swirling the melted agarose.

2.5.2. Talent adding agarose to a few wells.

2.5.3. Talent adding DMEM on top of the agarose.

2.6. Place the lid with the spheroid droplets back on top of the plate so that the spheroids are again hanging **[1]**, then centrifuge the plate for 3 minutes at 200 x *g* to transfer the spheroids into the individual wells of the plate **[2]**. Incubate the plate for another 24 hours to allow the spheroids to settle **[3]**. *Videographer: This step is important!*

2.6.1. Talent placing the lid back on top of the plate.

2.6.2. Talent putting the plate in the centrifuge and closing the lid.

2.6.3. Talent putting the plate back in the incubator.

3. Nanomaterial/Chemical Exposure and Spheroid Harvesting

3.1. Following dispersion of the engineered nanomaterials, or ENMs, dilute them to the final desired concentration with pre-warmed DMEM **[1-TXT]**. Aspirate 50 microliters of medium from each well with the spheroids **[2]** and replace it with 50 microliters of medium with the ENM **[3]**.

3.1.1. Talent diluting the ENMs. **TEXT: 5mL per 96-well plate**

3.1.2. Talent aspirating media from a few wells.

3.1.3. Talent adding medium with ENMs to a few wells.

3.2. To harvest the spheroids, use a 200-microliter pipette to aspirate the 100 microliters of cell culture medium with the spheroid tissue from each well, taking care to avoid contact with the agarose **[1]**. Collect the spheroids in a sterile, 15-milliliter centrifuge tube **[2]**. *Videographer: This step is difficult and important!*

3.2.1. Talent aspirating media from a well while avoiding the agarose.

3.2.2. Talent collecting media with spheroids in a tube.

3.3. Centrifuge the spheroid suspension at 230 x *g* for 5 minutes **[1]**, then remove the supernatant and store it at -80 degrees Celsius until further analysis **[2]**.

3.3.1. Talent placing the tube in the centrifuge and closing the lid. *Videographer: Obtain multiple usable takes, this will be reused in 3.4.2 and 3.5.2.*

3.3.2. Talent transferring supernatant to another tube.

3.4. Wash the pellet of spheroids in 1-milliliter of sterile, room temperature PBS **[1]**, then centrifuge them at 230 x *g* for 3 minutes and discard the supernatant **[2]**. Resuspend the spheroids in 500 microliters of 0.05% trypsin-EDTA solution **[3]** and incubate them for 6 to 8 minutes at 37 °C and 5% carbon dioxide **[4]**.

3.4.1. Talent adding PBS to the tube, with the PBS container in the shot.

3.4.2. *Use 3.3.1.*

3.4.3. Talent resuspending spheroids in Trypsin-EDTA, with the Trypsin container in the shot.

3.4.4. Talent putting the tube in the incubator and closing the door.

3.5. After the incubation, gently pipette the trypsinized HepG2 cells up and down to fully disassociate and resuspend them prior to neutralizing with 1 milliliter of DMEM **[1]**. Centrifuge the cell suspension at 230 x *g* for 5 minutes **[2]**, discard the supernatant **[3]** and resuspend the cell pellet in 2 milliliters of PBS **[4]**.

3.5.1. Talent pipetting the cells up and down, then adding the DMEM.

3.5.2. *Use 3.3.1.*

3.5.3. Talent discarding the supernatant.

3.5.4. Talent resuspending the cells in PBS.

4. Micronucleus Assay and Scoring

4.1. To create a cuvette funnel setup, place the prepared microscope slide into the metal support, place a filter card on top of the slide, then secure the cuvette funnel on top **[1]**.

4.1.1. Talent assembling the cuvette funnel setup.

4.2. Arrange the cuvette funnels in the cytocentrifuge with the funnel facing up, so that 100 microliters of cell suspension can be directly added into each one. Then, proceed with fixing the slides according to manuscript directions **[1]**.

4.2.1. Talent arranging the cuvette in the cytocentrifuge.

4.3. Prepare a 20% Giemsa staining solution diluted in phosphatase buffer **[1-TXT]**. Gently pipette the solution up and down to mix it, then filter it with folded filter paper in a funnel **[3]**.

4.3.1. Talent preparing the Giemsa solution. **TEXT: Caution! Giemsa staining solution is toxic and flammable**

~~4.3.2. Talent swirling the solution.~~

4.3.3. Talent filtering the solution.

4.4. Use a Pasteur pipette to add 3 to 5 drops of filtered Giemsa solution to the cytodot on each slide and leave it for 8 to 10 minutes at room temperature **[1]**. Wash the slides in two successive phosphatase buffer washes **[2]**, then briefly rinse them under cold water to remove any excess stain and leave them to air dry **[3]**.

4.4.1. Talent swirling and adding solution to a slide.

4.4.2. Talent washing the slide.

4.4.3. Talent rinsing the slide.

Results

5. Results: Morphology and Characteristics of HepG2 Spheroids

5.1. Prior to any in vitro toxicological assessment, it is important to check that the 3D HepG2 *(pronounce 'hep-G-2')* spheroids have formed properly **[1-TXT]**. Two days post-seeding, compact, spherical shaped spheroids with a smooth surface and no visual projections should form **[2]**.

5.1.1. LAB MEDIA: Figure 5. *Video Editor: Label A – C "***Day 2***", D – F "***Day 4 Good Quality***", and G – I "***Day 4 Bad Quality***".*

5.1.2. LAB MEDIA: Figure 5. *Video Editor: Emphasize A – C.*

5.2. Good quality **[1]** and poor quality **[2]** spheroids four days post-seeding are demonstrated here. Typically, 90 to 95% of spheroids formed per plate will form correctly and be viable for further experimentation **[3]**.

5.2.1. LAB MEDIA: Figure 5. *Video Editor: Emphasize D – F.*

5.2.2. LAB MEDIA: Figure 5. *Video Editor: Emphasize G – I.*

5.2.3. LAB MEDIA: Figure 5.

5.3. Viability and liver-like functionality was assessed over a 14-day culture period to determine the longevity of the liver spheroid model and establish if it could support long-term ENM or chemical based hazard assessment **[1]**.

5.3.1. LAB MEDIA: Figure 6.

5.4. Albumin concentration remained consistent over the duration of the culture period **[1]**, while urea production per spheroid showed an increase before reaching a plateau at day 7 **[2]**.

5.4.1. LAB MEDIA: Figure 6. *Video Editor: Emphasize B.*

5.4.2. LAB MEDIA: Figure 6. *Video Editor: Emphasize C.*

5.5. For genotoxicity assessment, the micronucleus assay was used to determine the presence of micronuclei following acute and long-term ENM exposures **[1-TXT]**.

5.5.1. LAB MEDIA: Figure 7. *Video Editor: Label the Acute Exposure data "***24 hours***" and Long-term Exposure "***120 hours***".*

5.6. The spheroids were exposed to two ENMs, titanium dioxide and sliver **[1]**. A similar trend for genotoxicity was observed after acute exposure to both ENMs **[2]**, but the elevated genotoxicity response was not evident after a long-term, 5-day exposure **[3]**.

5.6.1. LAB MEDIA: Figure 7.

5.6.2. LAB MEDIA: Figure 7. *Video Editor: Emphasize the Ag and TiO2 acute exposure bars.*

5.6.3. LAB MEDIA: Figure 7. *Video Editor: Emphasize the Ag and TiO2 long-term exposure bars.*

Conclusion

6. Conclusion Interview Statements

6.1. **Samantha V. Llewellyn:** Following this method, the spheroids and supernatants harvested can be used for a multitude of biochemical endpoints, including cell viability and liver function assays, CYP450 analysis, (pro-)inflammatory markers and gene expression.

6.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

6.2. **Samantha V. Llewellyn:** This technique is now being tested by a number of Contract Research Labs who want to apply it to more routine genotoxicity testing of both chemicals and nanomaterials. This will provide a bank of data that can potentially reduce the reliance on in vivo hazard testing approaches.

6.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.