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Title: In Vitro Quantitative Imaging Assay for Phagocytosis of Dead Neuroblastoma Cells by iPSC-Macrophages

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

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

Current Protocol Length

Number of Steps: 22
Number of Shots: 53

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Hazel Hall-Roberts:** This in vitro imaging assay makes it possible to quantify the effects of different treatments or different genotypes upon microglial phagocytosis, using two cell models that are relevant to neurodegenerative disease.

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

1.2. **Hazel Hall-Roberts:** We use dead neuroblastoma cells for the phagocytic cargo, which are prepared in a way that could be easily and cheaply scaled up for large high-content imaging screens.

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

Ethics Title Card

1.3. The protocol follows the guidelines for the use of human iPS cell lines derived at the University of Oxford, Oxford Parkinson's Disease Centre.

Protocol

2. Preparation of dead SH-SY5Ys

2.1. In a class II *('2')* biological safety cabinet, dissociate SH-SY5Ys *(S-H-S-Y-five-Ys)* by aspirating the media **[0].** Add 4 milliliters of cell dissociation buffer and remove the buffer immediately so that less than 1 milliliter remains as a thin film coating the cells **[1-TXT].** NOTE: Shots 2.1.0 and 2.1.1 are combined shot

2.1.0. Added SHOT: Talent aspirating medium.

2.1.1. Talent adding cell dissociation buffer to SH-SY5Ys cells. **TEXT: SH-SY5Ys - human neuroblastoma cell line.** Talent removing the buffer leaving only 1 ml of the buffer.

2.2. Incubate for 2 to 3 minutes at 37 degrees Celsius and 5% carbon dioxide **[1].** Then, add 10 milliliters of HBSS to the T75 *(T-seventy-five)* flask **[2-TXT]** and pipette the SH-SY5Ys into a 15-milliliters conical centrifuge tube **[3]**. Centrifuge at 400 times *g* for 5 minutes **[4].**

2.2.1. Talent incubating the cells.

2.2.2. Talent adding HBSS to the T75 flask. **TEXT: HBSS- Hanks' Balanced Salt Solution**

2.2.3. Talent pipetting the SH-SY5Ys into centrifuge tube.

2.2.4. Talent centrifuging the tube.

2.3. Aspirate the supernatant **[1]** and re-suspend the cells in 2 milliliters of phenol red-free HEPES-buffered media, making sure to break up clumps before fixation **[2]**.

2.3.1. Talent discarding the supernatant.

2.3.2. Talent adding phenol red-free HEPES-buffered media into the pellet and triturating. NOTE: Recorded incorrectly as 2.3.1, slightly out of focus, only one take possible as cells could not be respun

2.4. Fix the cells by adding 2 milliliters of 4% paraformaldehyde to the tube and incubating for 10 minutes at room temperature with occasional gentle agitation **[1].**

2.4.1. Talent adding 4% paraformaldehyde to the cells.

2.5. Add 10 milliliters of HBSS to the tube **[1]**, then centrifuge at 1,200 times *g* for 7 minutes **[2].** Aspirate the supernatant and re-suspend the cell pellet in 2 milliliters of phenol red-free HEPES-buffered media **[3].**

2.5.1. Talent adding HBSS to the tube.

2.5.2. Talent centrifuging the tube.

2.5.3. Talent aspirating the supernatant and adding phenol red-free HEPES-buffered media to the pellet.

3. Labeling of dead SH-SY5Ys with pH-sensitive red fluorescent dye

3.1. Count and remove one million SH-SY5Y cells into a 2-milliliter low protein-binding tube **[1].** Bring the total volume to 300 to 500 microliters with phenol red-free HEPES-buffered media **[2]**, then briefly warm the tube in a 37-degree Celsius water bath **[3].**

3.1.1. Talent transferring the cells into a 2 mL low-protein binding tube.

3.1.2. Talent adding phenol red-free HEPES buffered media.

3.1.3. Talent warming the tube in water bath.

3.2. Reconstitute the pH-sensitive red fluorescent dye STP ester according to manufacturer's instructions, then add 12.5 micrograms of dye per million SH-SY5Y cells **[1-TXT]**. Mix gently by flicking the tube **[2]** and incubate the tube at room temperature for 30 minutes, protected from light **[3].**

3.2.1. Talent adding red fluorescent dye to the tube. **TEXT: STP- 4-Sulfotetrafluorophenyl**

3.2.2. Talent mixing the tube.

3.2.3. Talent covering the tube with foil and incubating the tube at room temperature.

3.3. Add 1 milliliter of HBSS **[1]** and centrifuge at 1200 times *g* for 7 minutes and 4 degrees Celsius **[2]**. Discard the supernatant **[3]** and wash with 2 milliliters of HBSS **[4].** Centrifuge again with the same settings **[5]**.

3.3.1. Talent adding 1mL HBSS to the tube.

3.3.2. Talent centrifuging the tube.

3.3.3. Talent discarding the supernatant.

3.3.4. Talent adding 2 mL HBSS to the tube. NOTE: Combined shot with 3.3.5

3.3.5. Added: Talent centrifuging the tube. NOTE: Shot 3.3.4 is split into two, this shot contains the centrifuge action

3.4. Re-suspend the cell pellet in phenol red-free macrophage media to a concentration of 0.2 to 1.2 million cells per milliliter, so that 50 microliters contain 10,000 to 60,000 cells **[1].**

3.4.1. Talent discarding the supernatant and adding phenol red-free macrophage media to the cells.

4. Staining of iPSC-macrophages

4.1. In a biological safety cabinet, prepare a solution of a deep red-fluorescent, cell-permeant, succinimidyl ester-reactive dye in macrophage media **[1]**. Add Hoechst *(pronounce 'hookst')* 33342 **[2]** and warm the working solution to 37 degree Celsius in a water bath **[3].**

4.1.1. Talent preparing ester-reactive dye in macrophage media.

4.1.2. Talent adding Hoechst dye.

4.1.3. Talent placing the working solution in water bath.

4.2. Aspirate the iPSC-macrophage *(I-P-S-C)* medium gently by pipetting the cell supernatant with a multichannel pipette into a sterile reservoir **[1-TXT]**. Add 70 microliters per well of the dye solution to the iPSC-macrophages, using a multichannel pipette **[2]**. Incubate for 1 hour at 37 degrees Celsius and 5% carbon dioxide **[3].**

4.2.1. Talent aspirating the iPSC-macrophage medium into a sterile reservoir. **TEXT: iPSC- an induced pluripotent stem cell**

4.2.2. Talent adding prepared dye to iPSC-macrophages.

4.2.3. Talent incubating the plate at 37 degrees. NOTE: Shots repeated for 4.4.3 and 5.1.2

4.3. Prepare experimental treatments in phenol red-free macrophage media **[1-TXT]**. After the incubation, aspirate the iPSC-macrophage medium very gently with a multichannel pipette **[2]** and add 100 microliters of HBSS per well **[3]**.

4.3.1. Talent preparing experimental treatment. **TEXT: 10 µM cytochalasin D as negative control**

4.3.2. Talent aspirating the iPSC-macrophage medium into a sterile reservoir.

4.3.3. Talent adding HBSS to wash (from reservoir labelled “HBSS”).

4.4. Immediately remove HBSS by gentle pipetting **[1]**, then add 100 microliters of phenol red-free macrophage media, plus experimental treatments in separate wells **[2]**. Incubate for 10 minutes to 1 hour at 37 degrees Celsius and 5% carbon dioxide **[3].**

4.4.1. Talent removing the HBSS solution into a sterile reservoir.

4.4.2. Talent adding media into a row of the plate (from reservoir labelled “media”) and cytochalasin D treatment (from separate reservoir labelled “cytoD”) into another row of the plate.

4.4.3. Talent placing the plate in an incubator. NOTE: Action captured in shot 4.2.3

5. Fixed-cell high-content imaging

5.1. Use a multichannel pipette to add 50 microliters of the labeled SH-SY5Ys per well, from the sides of each well at the edge of the liquid **[1]**, then incubate at 37 degrees Celsius and 5% carbon dioxide for 3 to 5 hours **[2].**

5.1.1. Talent adding labeled SH-SY5Ys to the plate

5.1.2. Talent placing the plate in an incubator. NOTE: Action captured in shot 4.2.3

5.2. After the phagocytosis incubation, gently aspirate cell supernatants by pipetting with a multichannel pipette and discard **[1]**. Wash once with 100 microliters of PBS **[2]**, then fix the cells by adding 100 microliters of 2% paraformaldehyde and incubating for 15 minutes at room temperature **[3].**

5.2.1. Talent discarding the cell supernatant.

5.2.2. Talent adding PBS to the cells and then aspirating into a reservoir.

5.2.3. Talent adding 2% paraformaldehyde to the cells.

5.3. Aspirate wells and add 100 microliters of PBS **[1,2]**. Either proceed directly to imaging with the high-content microscope **[3]** or cover with plate sealer and foil and store the plate at 4 degree Celsius until required **[4].**

5.3.1. Talent aspirating from the wells.

5.3.2. Talent adding PBS to the wells. NOTE: Shot combined with 5.3.1

5.3.3. Talent placing the plate into the high-content microscope for imaging.

5.3.4. Talent covering the plate with sealer and foil for storage.

5.4. Turn on the high-content imaging microscope and open the image capture software. Load the assay plate into the microscope by clicking on the **Load** icon at the top of the screen **[1].**

5.4.1. SCREEN: 62217_screenshot_1.mp4. 00:03-00:38. *Video editor speed up the video.*

5.5. Select the **Setup** tab. In the drop-down menus of the top left box, select the appropriate plate type, the autofocus option **Two Peak**, the objective **40x Water, NA1.1**, **Confocal** mode, and binning of **1 [1].** Flush the 40x water objective before use via the **Settings** menu **[2].**

5.5.1. SCREEN: 62217_screenshot_2.mp4. 00:03-00:20.

5.5.2. SCREEN: 62217_screenshot_3.mp4. 00:02-00:35. *Video editor speed up the video.*

5.6. In the **Channel Selection** box, use the plus icon to add the channels DAPI, Alexa 647, and Alexa 568. Set these to measure at a single plane of 1 micrometer **[1]**. Optimize **Time** and **Power** settings for the staining efficiency of the assay plate **[2].**

5.6.1. SCREEN: 62217_screenshot_4.mp4. 00:01-00:24.

5.6.2. SCREEN: 62217_screenshot_5.mp4. 00:01-00:27.

5.7. Ensure the channels are not measured simultaneously by clicking on **Channel Sequence** to separate out the channels**.** Under **Navigation** and **Define Layout**, select the wells of measurement and select 9 to 12 fields per well **[1].**

5.7.1. SCREEN: 62217_screenshot_6.mp4. 00:01-00:46. *Video editor speed up the video.*

5.8. During set-up, click on a representative field on the plate map, and check each measurement channel in turn to ensure that the staining is present and that the images are focused by adjusting the channel offset **[1].**

5.8.1. SCREEN: 62217_screenshot_7.mp4. 00:02-00:26*Video editor speed up the video.*

5.9. To upload the data to a server for remote analysis, click on the **Online Jobs** box and the relevant screen name. Save the assay protocol by clicking on the **Save** button and click on the **Run Experiment** tab at the top. Name the experiment plate, then click on **Start [1].**

5.9.1. SCREEN: 62217_screenshot_8.mp4. 00:02-00:32.

Results

6. Imaging, optimization and validation of phagocytosis assay

6.1. A live-cell time-lapse phagocytosis assay showed that with 10,000 SH-SY5Ys per well, the number of phagocytosed particles per cell increased linearly with time and was inhibited by approximately 50% by cytochalasin D **[1].**

6.1.1. LAB MEDIA: Figure 3A. *Video editor focus on the green spots.*

6.2. With higher amounts of SH-SY5Ys per well, phagocytosis exhibited poor linearity, likely due to poor segmentation of iPSC-macrophages and SH-SY5Ys in a more crowded field of view **[1].**

6.2.1. LAB MEDIA: Figure 3B and 3C.

6.3. This representative high-content microscopy image of SH-SY5Ys phagocytosed by wild-type iPSC-macrophages was obtained by performing fixed-cell high-content imaging **[1].** Increasing the amount of SH-SY5Ys resulted in a higher number of phagocytosed particles per cell **[2].**

6.3.1. LAB MEDIA: Figure 4A.

6.3.2. LAB MEDIA: Figure 4B.

6.4. The phagocytosis assay was validated using several inhibitors of phagocytosis **[1].** Cytochalasin D and jasplakinolide significantly inhibited phagocytosis by 91 and 90%, respectively **[2]**, and bafilomycin A1 significantly reduced phagocytosis by 31% when pre-incubated for 1 hour prior to phagocytosis **[3].**

6.4.1. LAB MEDIA: Figure 4C.

6.4.2. LAB MEDIA: Figure 4C. *Video editor focus on the graph of Cyt and Jas showing red and orange spots, respectively.*

6.4.3. LAB MEDIA: Figure 4C. *Video editor focus on the graph of Baf showing green spots.*

6.5. Addition of recombinant annexin V *('five')* significantly reduced phagocytosis by 30%, when added to wells immediately before SH-SY5Y addition **[1]**.

6.5.1. LAB MEDIA: Figure 4C. *Video editor focus on the graph of Ann showing purple spots.*

6.6. Fixed SH-SY5Ys were confirmed to expose phosphatidylserine using a fluorescent annexin V probe **[1]**, whereas live SH-SY5Ys were negative for annexin V staining **[2].**

6.6.1. LAB MEDIA: Figure 4D. *Video editor focus on the 2% PFA-fixed SH-SY5Ys cells.*

6.6.2. LAB MEDIA: Figure 4D. *Video editor focus on the live SH-SY5Ys cells.*

6.7. Several lengths of phagocytosis duration from 1 to 5 hours were tested using staggered addition of phagocytic cargo **[1].**

6.7.1. LAB MEDIA: Figure 5.

Conclusion

7. Conclusion Interview Statements

7.1. **Hazel Hall-Roberts**: The overall length of the protocol can be shortened by preparing the phagocytic cargo and staining the iPSC-macrophages in parallel. If you want to do this, work out the timings in advance and use incubations to prepare solutions for the future steps. NOTE: Shot 2 or shot 5, may be *noises off* at end of shot

7.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.4.2.*