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Title: Automated Production of Human Induced Pluripotent Stem Cell-Derived Cortical and Dopaminergic Neurons with Integrated Live-Cell Monitoring

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Author Questionnaire

1. Microscopy: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **N**

2. Software: Does the part of your protocol being filmed demonstrate software usage? **Y**

3. Filming location: Will the filming need to take place in multiple locations (greater than walking distance)? **N**

Protocol Length
Number of Shots: **55**

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Ashutosh Dhingra**: The overall goal of this study is to show how to implement automated procedures for culturing and differentiating –human iPS cells to neuronal lineages as well as their automated imaging **[1]**.

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

REQUIRED:

1.2. **Ashutosh Dhingra**: Standardized automated procedures -allow low experimental variation while ensuring high phenotypic reproducibility. In addition, this system can be adapted for the development of new cell culture protocols **[1]**.

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

Introduction of Demonstrator on Camera

1.3. **Ashutosh Dhingra**: Demonstrating the procedures will be Joachim Taeger and Elisangela Bressan, post docs from our lab **[1][2]**.

1.3.1. INTERVIEW: Author saying the above
1.3.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera

Protocol

2. New Culture Plate and Tip Loading and Decontamination

2.1. In the graphic user interface of the automated system, click **Resource-Instrument process view [1]** and select the resource-instrument process **[2]**.

2.1.1. WIDE: Talent clicking button, with monitor visible in frame

2.1.2. SCREEN: screensho_1: 00:00-00:02

2.2. Click **Run Instrument process** and run the **RunHepaHood** and **Reloading** processes **[1-TXT]**.

2.2.1. SCREEN: screensho_1: 00:03-00:09 **TEXT: Monitor material placement to avoid transport errors**

2.3. Select the resources to be loaded **[1]**, open the door **[2]**, and load the cell culture plates and disposable tips in the appropriate positions as indicated by the pop-up images **[3]**.

2.3.1. SCREEN: screenshot_3: 00:00-00:10

2.3.2. Talent opening door

2.3.3. Talent loading plate(s) and/or tips

2.3.4. SCREEN: screenshot_3: 00:11-00:23

2.4. Then decontaminate the door with 70% ethanol before closing **[1-TXT]** and run the **Decontamination** process. The system will be sterilized by UV radiation for 30 minutes **[2]**.

2.4.1. Talent spraying and/or wiping door **TEXT: Decontaminate door after every handling**

2.4.2. SCREEN: screensho_4: 00:00-00:08

3. Culture Medium and/or Dissociation Reagent Filling

3.1. To load the plates with culture medium or dissociation reagent, execute the **RunHepaHood** step **[1]** and open the door of the liquid handling station **[2]**.

3.1.1. WIDE: Talent executing step, with monitor visible in frame

3.1.2. Talent opening door

3.2. After decontamination with 70% ethanol **[1]**, place the reservoirs on the deck to the assigned position **[2]** and de-lid them **[3]**.

3.2.1. Talent spraying/wiping reservoirs with ethanol

3.2.2. Talent placing reservoir(s) onto deck

3.2.3. Talent removing lid(s)

3.3. Then run the **InitHepaHood** resource-instrument process to power down the HEPA hood **[1]**.

3.3.1. SCREEN: screenshot_2: 00:00-00:11

4. Automated Method Execution

4.1. To execute an automated culture method, in the calendar view of the graphic user interface **[1]**, click **Add process step** and select the cell line to be used in the experiment **[2]**.

4.1.1. WIDE: Talent selecting calendar view, with monitor visible in frame

4.1.2. SCREEN: screensho_5: 00:00-00:07

4.2. Use the wizard to select the project and mark the batch to be used. Click the right-facing arrowhead and navigate to the next page of the wizard to select the process step to be executed **[1-TXT]**.

4.2.1. SCREEN: screenshot_5: 00:07-00:14 **TEXT: Batch should be empty or contain culture or assay plates**

4.3. In the last page of the wizard, schedule the experiment and set the appropriate **Parameter Details** variables necessary for running the method. Then click **OK [1]**.

4.3.1. SCREEN: screensho_5: 00:14-00:24

5. Human Induced Pluripotent Stem Cell (hiPSC) Seeding

5.1. After loading a 50-milliliter tube with the human iPS (eye-P-S-) cell suspension **[1]**, load coated culture plates for receiving cells on the shelf **[2-TXT]** and execute **the Seeding of plates from tubes** method **[3]**.

5.1.1. WIDE: Talent loading tube into system, with monitor visible in frame

5.1.2. Talent loading plate(s) onto shelf **TEXT: Decontaminate and close doors after loading**

5.1.3. SCREEN: screenshot_6: 00:12-00:20

5.2. To count the cells in the brightfield imaging cytometer, automatically prepare a 384-well counting plate with cell suspension on the deck ~~**[1]**~~ and use the robotic arm to transfer the 384-well counting plate from the deck to the brightfield imaging cytometer **[2-TXT]**.

5.2.1. Author NOTE: automatic function so not shot.

5.2.2. Arm transferring plate to cytometer **TEXT: cytometer automatically determines the cell number per milliliter**

5.3. ~~**[1-TXT]**~~.

5.4. After counting, the system will return the plate to its original position **[1]** and transfer a coated culture or assay plate from the shelf to the pipetting deck **[2]**.

5.4.1. Arm returning plate *Videographer: Important step*

5.4.2. Arm selecting/transferring plate to pipetting deck *Videographer: Important step*

5.5. The system will remove coating and add fresh medium **[2-TXT]** . The system will then seed the cells in the user-defined number and volume suitable for the plate **[1]**.

5.5.1. Cells being seeded

5.5.2. The coating is removed and add fresh medium **TEXT: Remove coating before cell seeding as necessary.** NOTE: Please move this step before 5.5.1

5.6. After seeding, the plate will be moved to the on-deck shaker for 10 seconds at 500 revolutions per minute for cell distribution **[1]** before being transferred to the carbon dioxide incubator **[2]**.

5.6.1. Shot of plate on shaker

5.6.2. Plate being placed into incubator

6. Automated Confluence Assessment

6.1. To assess the automated confluence, execute the **Check Confluency** method **[1]** and select a batch that contains at least one culture plate and no assay plates **[2]**.

6.1.1. WIDE: Talent executing method, with monitor visible in frame

6.1.2. SCREEN: screenshot_8: 00:06-00:12

6.2. In the **Parameter Details** section, enter iPSCf_2020 for image acquisition and the imaging analysis settings **[1]**.

6.2.1. SCREEN: screenshot_8: 00:16-00:29 *Video Editor: can speed up*

6.3. The system will then transfer the first plate from the incubator to the brightfield imaging cytometer **[1]** and image the cell confluence **[2-TXT]**.

6.3.1. Plate being transferred with robotic arm *Videographer: Important step*

6.3.2. Cells being imaged *Videographer: Important step* **TEXT: Transfer plate back to incubator after imaging**

7. Medium Change

7.1. To change cell culture or assay plate media, execute the **Media Change of Culture Plates** method **[1]** and select a batch containing only culture plates **[2]**.

7.1.1. WIDE: Talent executing method, with monitor visible in frame

7.1.2. SCREEN: screenshot_9: 00:06-00:13

7.2. The system will transfer the plates to the deck **[1]** and tilt the plates to allow aspiration of the supernatant **[2]**.

7.2.1. Arm moving plate(s) to deck *Videographer: Important step* NOTE: included in shot 6.3.1 take 6).

7.2.2. Tilting plate and/or supernatant being aspirated *Videographer: Important step*

7.3. The supernatant will be discarded into the waste collection module **[1]** and 12 milliliters of fresh medium will be added to each plate **[2]**.

7.3.1. Arm discarding supernatant into waste module and discarding used tips *Videographer: Important step*

7.3.2. Arm adding medium to well(s) *Videographer: Important step*

7.4. Then system will then re-lid the plates **[1]** and return the plates to the cell culture incubator **[2]**.

7.4.1. Arm putting lid onto plate

7.4.2. Arm placing plate into incubator

7.5. Alternatively, to change medium of assay plates, execute Media Change of Assay Plates method. **[1]**

7.5.1. Media change of 96-well assay plates. (shot of 96-well assay plates on the deck with pipette tips). NOTE: Maybe added shot

8. Subcultivation

8.1. To subcultivate the cells, execute the **Subcultivation of Adherent Cells** method **[1]** and select the batch containing the culture plates that need subcultivation **[2]**.

8.1.1. WIDE: Talent selecting method, with monitor visible in frame

8.1.2. SCREEN: screenshot_10: 00:09-00:16

8.2. After discarding the medium, the system will wash the cells one time with 8 milliliters of PBS per plate **[1]** before adding 8 milliliters of 0.5-millimolar EDTA to the cells **[2]**.

8.2.1. Arm adding PBS to cells

8.2.2. Arm adding EDTA to cells

8.3. After 8 minutes on the deck with tilting option **[1]**, the EDTA will be replaced with 12 milliliters of fresh medium per plate **[2]**.

8.3.1. Aspirate EDTA and discard

8.3.2. Arm adding medium

8.4. The system will shake the plates at 2000 revolutions per minute for 1 minute to dislodge the colonies **[1]** before triturating the cells with five cycles of pipetting to break up the colonies into 50-80-micron clumps **[2]**.

8.4.1. Plates being shaken OR Plates being transferred to shaker

8.4.2. Cells being triturated

8.5. Then cells will then be transferred to a 50-milliliter tube on the deck **[1]** before seeding them in a 1:7 split ratio **[2-TXT]**

8.5.1. Arm adding cells to tube

8.5.2. Arm adding cells to plate. **TEXT: Transfer plates to the incubator**

9. Automated High Content, High Throughput Imaging

9.1. For automated, high content, high throughput cell imaging, execute the **Imaging** method **[1]** and select a batch that contains at least one assay plate and no culture plates **[2]**.

9.1.1. WIDE: Talent executing method, with monitor visible in frame

9.1.2. SCREEN: screenshot_11: 00:08-00:11

9.2. Then system will then transfer an assay plate to the automated confocal microscope for imaging **[1-TXT]**.

9.2.1. Arm transferring plate *Videographer: Important step* **TEXT: Initiate plate return to incubator after imaging**

Protocol Script Questions

A. Which steps from the protocol are the most important for viewers to see?

5.4., 6.3., 7.2., 7.3., 9.2.

B. What is the single most difficult aspect of this procedure and what do you do to ensure success?

2.2. Reloading of culture plates or other consumables. Please pay attention to proper alignment of the plates/ tips racks while loading. Misplacements can cause transport error and thus abort the method and subsequent steps.

Results

10. Results: Representative Automated System-Produced hiPSC-Derived Cortical and Dopaminergic Neuron Characterization

10.1. The hiPSC cultures should be monitored daily for growth **[1]** and analyzed for their percentage of confluency in the brightfield imaging cytometer **[2]**.

10.1.1. LAB MEDIA: Figure 3 *Video Editor: please sequentially emphasize left and right Figure 3A images*
10.1.2. LAB MEDIA: Figure 3 *Video Editor: please emphasize Figure 3B*

10.2. After passaging and manual or automated system culture **[1]**, the cells exhibit a typical stem cell **[2]** and pluripotency marker expression **[3]**.

10.2.1. LAB MEDIA: Figure 4A *Video Editor: please sequentially emphasize left and right images*
10.2.2. LAB MEDIA: Figures 4B and 4C *Video Editor: please emphasize OCT4 and SEEA4 images*
10.2.3. LAB MEDIA: Figures 4B and 4C *Video Editor: please sequentially emphasize graphs in Figure 4C*

10.3. Neurons differentiated in the automated culture system **[1]** demonstrate a similar morphology and neuronal network organization **[2]** to neurons cultivated manually **[3]**.

10.3.1. LAB MEDIA: Figure 5A
10.3.2. LAB MEDIA: Figure 5A *Video Editor: please emphasize Automated image*
10.3.3. LAB MEDIA: Figure 5A *Video Editor: please Manual image*

10.4. After 6 days of differentiation, automatically differentiated cortical neurons **[1]** are positive for neuron-specific class three beta-tubulin **[2]** and upper cortical layer marker expression **[3]**.

10.4.1. LAB MEDIA: Figure 5B
10.4.2. LAB MEDIA: Figure 5B *Video Editor: please emphasize TUBB3 image*
10.4.3. LAB MEDIA: Figure 5B *Video Editor: please emphasize BRN2 image*

10.5. After 8 days, the cells also express **[1]** microtubule-associated protein 2 **[2]**, the neural cell adhesion molecule **[3]**, and Synapsin-1 **[4]**, as well as cortical neuron markers **[5]**.

10.5.1. LAB MEDIA: Figure 5C
10.5.2. LAB MEDIA: Figure 5C *Video Editor: please emphasize MAP2 graph*
10.5.3. LAB MEDIA: Figure 5C *Video Editor: please emphasize BRN2 graph*
10.5.4. LAB MEDIA: Figure 5C *Video Editor: please SYN1 graph*
10.5.5. LAB MEDIA: Figure 5C *Video Editor: please CUX2 and NCAM1 graphs*

10.6. Very low or no expression of these markers is observed in hiPSC **[1]**.

10.6.1. LAB MEDIA: Figure 5C

10.7. The system can also be used to establish a live-cell automated neurite outgrowth assay **[1]** that allows neurite lengths to be measured over 11 days of differentiation without manual intervention **[2]**.

10.7.1. LAB MEDIA: Figures 6A and 6B *Video Editor: please sequentially add Figure 6B images under Figure 6A images*
10.7.2. LAB MEDIA: Figure 6C *Video Editor: please emphasize data lines or no animation*

10.8. Using the automated culture system to perform medium changes over a 65-day culture period **[1]** facilitates hiPSC differentiation into midbrain dopaminergic neurons with the expected cellular organization and morphology **[2]**.

10.8.1. LAB MEDIA: Figure 7
10.8.2. LAB MEDIA: Figure 7 *Video Editor: please sequentially emphasize Figures 7B, 7C, and 7D*

Conclusion

11. Conclusion Interview Statements

11.1. **Ashutosh Dhingra**: Be sure to select the correct batches and plate ids when performing the required steps **[1]**.

11.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (6.1., 7.1., 8.1., 9.1.)

11.2. **Elisangela Bressan**: In addition to the neurite-outgrowth assay, other automated phenotypic assays relevant to neurodegeneration, for example, TDP-43 translocations, RNA foci, and fibril uptake, can be explored using this high-throughput, high-content format **[1]**.

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