#### Standard Operating Procedure-Worksheet

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| Title: | SPARK WSOP #2 | | Version | 01 |
| Date: | 03/25/2019 | | Version Date | 03/25/2019 |
| Author: | David E. Solow-Cordero | | Project Code: | 223-02 |
| Performed by: | |  | | |
| Date Performed: | |  | | |
| Library & Number of plates: | |  | | |
| Plate #s: | |  | | |

**Purpose:** To screen for compounds that inhibit or activate MEGF10 to correct aberrant astrocyte-mediated synaptic pruning in schizophrenia.

**Assay Description**: We seek to identify small molecule inhibitors of the MEGF10 pathway, a mediator of astrocyte synaptic phagocytosis, as a treatment for aberrant synaptic pruning in diagnosed SZ patients post first psychotic break. We also seek to identify small molecule activators of the MEGF10 pathway as a treatment for dysregulated or absent synaptic pruning in the 22q11.2 deletion-associated SZ.

Target rationale:

* Postmortem analysis of brains of generalized schizophrenia patients show synapse loss; analysis of brains of 22q11.2 deletion syndrome with SZ demonstrate opposite effect
* Evidence of astrocytosis in SZ, as measured by GFAP ex vivo
* Genetically defined subset of SZ patients demonstrate 2X over-expression of MEGF10
* MEGF10 has been shown to be necessary and sufficient for astrocyte-mediated synaptic pruning *in vitro*
* MEGF10 is expressed selectively on astrocytes, limiting potential for on-target off-tissue toxicity

The screen will be a phagocytosis assay using astrocytes isolated from fetal human brain samples and synaptosomes prepared from mouse brain samples. Phagocytosis will be measured with a pH-sensitive fluorescent dye (pHrodo red) conjugated to the synaptosomes that is only activated when engulfed and localized to the low pH found in intracellular lysosomes. On day 0, 1000 astrocytes/well will be plated into 384-well plates in 50L. After 3 days at 37oC, 10% CO2, the Pin Tool on the SciClone ALH3000 will add 200 nL of compounds to the plates. Then 40L of additional media with 0.1% serum and 0.5L/well of pHrodo (pHrodo™ Red AM Intracellular pH Indicator) containing labeled synaptosomes will be added to columns 1-22. Columns 23-24 will receive just media. These synaptosomes will only fluoresce red when internalized in astrocytes. The plates will then be transferred to the Thermo Cytomat 2C incubator and after another 24 hours at 37oC, 10% CO2, the plates will be removed from the incubator, 10 L of Calcein AM (Fisher C3100MP) will be added and then the plates will be imaged in the IX Micro using the 4X objective, **BS\_Prodo\_calAM.HTS** protocol with the FITC-FIXED and TRITC-FIXED cubes. Image analysis protocols will be run and hits will be determined as compounds that increase or decrease the pHrodo signal relative to the Calcein AM signal compared to DMSO controls.

**Reagent List:**

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| --- | --- | --- | --- | --- | --- |
| **Reagent** | **CAS Number** | **Vendor- Manufacturer** | **Item Number** | **Lot Number**  **/Date Made** | **Misc.** |
| Astrocyte Media 50% DMEM 50% neurobasal | 292 ug/mL L-Glutamine | 5 ug/mL N-acetyl cysteine | 1x SATO | 1 mM Na+pyruvate | 100U/mL Pen/100ug/mL Strept |
| pHrodo Red AM  Ex 560 Em 585 |  | ThermoFisher | P35372 |  |  |
| Calcein, AM  Ex 495 Em 515 | 148504-34-1 | ThermoFisher | C3100MP |  | MW 994.87 |
| Pin Cleaning Solution |  | V&P Scientific | VP 110 | 30 mL & 120 mL ddH2O | Phosphoric Acid |
| Methanol | 67-56-1 | Fisher | A433P-4 | 032008-36 |  |

**Equipment & Materials List:**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Equipment Name** | **Vendor (Manufacturer)** | **Item Number/ Model Number** | **Lot/Serial Number** | **Misc.** |
| Twister II | CaliperLS | 79838/7 | T20407N0068 |  |
| SciCloneALH 3000 | CaliperLS | ALH3000 | SS0407R4317 |  |
| 384 Pin Tool 100 nL | V&P Scientific | AFIX384FP3H  Floating Tube, FP3 | BMPZYMARK | Hydrophobic pin 0.787 Diameter |
| ALHLow volume EZ-Swap head | CaliperLS | 103801 | SS0405N4294 |  |
| Oil Less Air Compressor | Jun-Air | 2xOF302-40B | 609826 | Velocity11 and Staccato |
| Microscan 710 Barcode Scanner | CaliperLS | 76709 | 0408957 |  |
| Centrifuge | Beckman | Allegra-6 | AL599317 |  |
| Multidrop 384 | Titertek | 5840200 | 832003819/32003965 | For adding media or Calcein AM |
| Multidrop 384 | LabSystems | 832 | 832000-108/441 | For adding media or Calcein AM |
| Stand Alone 198 position incubator | Liconix | 104283 | 3183BT |  |
| Black Clear-bottom 384 well plates | E&K Scientific (Greiner) | EK-30946 or 948 |  | PDL coated |
| WellMate Dispenser | Matrix | 201-10001 | 119542592 | For plating cells and synaptosomes |
| WellMate Stacker | Matrix | 201-20001 | 201-2-0107 |  |
| WellMate Tubing-Small Bore | Matrix | 201-30002 |  |  |
| Lint Free Blotting Media | V&P Scientific | VP 540D |  |  |
| Polypropylene pad | V&P Scientific | VP 540DB1 |  |  |
| Omni Tray | V&P Scientific | VP 540DB |  |  |
| PlateLoc | Velocity11 | 01867.001 | 1.00406 | For sealing compound plates |
| BenchCel R-Series 2-4 Stack | Agilent | 18692-204 | SGS10BCL25101 |  |
| Oil Less Air Compressor | Jun-Air | 2xOF302-40B | 717613 | Agilent System |
| ImageXpress Micro | Molecular Devices | IXMicro | IX122639 | <http://htbc.stanford.edu/equipment/imaging>.html |
| Catalyst Express Robot | ThermoFisher (Thermo CRS) | F01229 | RCE1008969 |  |
| CRS 5-Robot Arm | ThermoFisher (Thermo CRS) | F01064 | RA1012719 |  |
| Cytomat 2C, Automated Incubator | ThermoFisher (Thermo LED) | 2C | 40994166 |  |
| Micorscan Barcode Reader | ThermoFisher (Microscan) | MS-3 | 1025150 |  |
| Vacuum Pump Box | ThermoFisher (ThermoSci) | P0094 |  |  |
| Pump for De-lidder | ThermoFisher (Hanning) | SV1003 D | 090054532 |  |
| FITC-FIXED | Semrock | FF01-485/20 |  | Excitation Filter |
| FITC-FIXED | Semrock | FF01-525/30 |  | Emission Filter Cube |
| FITC-FIXED | Semrock | FF506 |  | Dichroic |
| TRITC-FIXED | Semrock | FF01-560/25 |  | Excitation Filter |
| TRITC-FIXED | Semrock | FF01-607/36 |  | Emission Filter Cube |
| TRITC-FIXED | Semrock | FF410\_504\_582\_669 |  | Quadband Dichroic |

**Procedures:**

### **Day 0**: Plating of astrocytes. Date:\_\_\_\_\_\_\_\_\_\_\_\_\_

### This step will add 50 L of astrocytes (1000 cells/well) to columns 1 to 23 of a 384-well plate, and 90 L media to column 24. This will be performed on **Day 0**.

|  |  |  |
| --- | --- | --- |
| Task Number | Check List | Action |
| 1. |  | Label solid-black 384 well Greiner plates PDL coated (EK-30946) with the bar code **SP** and then the date (year, month, day) and a serialized number (\_01 to \_42 or so). Use the Excel file “**spark.xls”** on the barcode printer computer to generate the barcode list and use the **WASP** software with the “**spark.lab**”template to print the barcodes. Apply the barcodes on the A1-P1 side. Example **SP180611\_01**. |
| 2. |  | Number of plates: Labels:  \_\_\_\_\_\_\_\_\_\_\_\_\_\_ \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| 3. |  | Using the WellMate with Stackers, set to **384**, **volume** to **90** and **columns** to columns **24** only. |
| 4. |  | Prime WellMate with media. |
| 5. |  | Remove lids from plates and place in Left Stacker (height to 13.5), and then press Start to fill column 24 with media. |
| 6. |  | Using the WellMate with Stackers, set to **384**, **volume** to **50** and **columns** to **1-23**. |
| 7. |  | Prime WellMate with astrocytes. |
| 8. |  | Return plates to Left Stacker (height to 13.5), and then press Start to fill columns 1-23 with cells. |
| 9. |  | After adding cells to plates, let sit at room temperature on lab bench for 15 to 30 minutes to let cells settle and attach. |
| 10. |  | Place in TC Incubator, Liconix automated incubator, or Thermo CytoMat 2C automated Incubator 37oC, 10% CO2. |
| 11. |  | Wash Wellmate tubing with 4X ddH2O, 4X EtOH, 2X ddH2O. |

### **Day 3**: Addition of compounds to cell plates: Date:\_\_\_\_\_\_\_\_\_\_\_\_\_\_

In this step the 384 Pin Tool to add about 200 nL of each compound to the cell plates. This will be performed immediately after adding synaptosomes.

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| --- | --- | --- |
| Task Number | Check List | Action |
| 1. |  | Set out compound plates to thaw at room temperature for 1 hour.  Library: # Plates:  \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| 2. |  | Plate Labels:  \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| 3. |  | Manually place the following on the Deck of Sciclone:  **A1:** Pin Tool  **B2:** Cleaning solution reservoir (fill with 150 mL)  **B4:** Cleaning solution Blotting Station (Lint Free Blotting Media on top of Polypropylene pad inside of Omni Tray)  **C2:** ddH2O reservoir (fill with 200 mL)  **C3:** ddH2O Blotting Station  **C4:** Methanol reservoir (fill with 200 mL, refill as needed-due to evaporation)  **C5:** Methanol Blotting Station  See Maestro Software “**384head.app” Layout(1).** |
| 4. |  | Make sure the **ALHLow volume EZ-Swap** head is loaded on the SciClone. |
| 5. |  | Check astrocytes under microscope to make sure they are still alive and well formed, 50-70% confluent. Make sure the plates are in the Liconic Incubator **Position 1**. |
| 6. |  | Remove foil on **compound plates** and place plates in the **Twister stack #1**and **#2** if more than 39 plates. First Plate on top. Barcodes facing out. |
| 7. |  | Open **iLink Pro**. Wait for resources to be created. May need to select “**Update**” on the AnalystGT computer. |
| 8. |  | Click on the “**Run Method**” Button. |
| 9. |  | Select Method “**2XCompAdd1XPos1.mth**” making sure the first cell plate is in position 1. This Method is located in the **CellBased** Folder. Click **Finish**. |
| 10. |  | Select **# of Runs** = number of cell plates.  Number of Runs:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| 11. |  | Click on the **INITIALIZE** button. Wait until everything is initialized and **RUN** is available. |
| 12. |  | Select the **RUN** button in **iLinkPro** to start the run.  Time:\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |
| 13. |  | During the run you may need to re-fill the Methanol reservoir. |
| 14. |  | At end of run, using the **Maestro Software**, run method “**CleanPins**”. Then unload the Pin Tool with the “**ParkPin**” method. |
| 15. |  | Exit the **iLink Pro** software. |
| 16. |  | Reseal compound plates with **Agilent System**. Place in BC-2 Stack 3 and run the **Seal 384.pro** (170oC, 1”) method in **VWorks** and return plates to -20oC. |
| 17. |  | Reuse Cleaning solution by pouring in Cleaning solution storage bottle and storing in Corrosives cabinet. Dispose ddH20 and Methanol. Cover all Omni trays for future use. Replace with fresh blotting material as needed. |
| 18. |  | Barcode File Name:  \_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_ |

### Addition of Synaptosomes. Date:\_\_\_\_\_\_\_\_\_\_\_\_\_

### This step will add 40 L of media containing 0.225% Serum and 0.5 L of pHrodo labeled Synaptosomes to columns 1 to 22, and 24 of a 384-well plate.

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| --- | --- | --- |
| Task Number | Check List | Action |
| 1. |  | Using the WellMate with Stackers, set to **384**, **volume** to **40** and **column** to **1-22** and **24**. |
| 2. |  | Prime WellMate with media and then Synaptosome containing media. |
| 3. |  | Remove lids from plates and place in Right Stacker (height to 13.5), and then press Start. |
| 4. |  | Can optionally add 40 uL of media only (with 0.225% Serum) to column 23. |
| 5. |  | Return plates to the Liconix automated incubator, or place in Thermo CytoMat 2C automated Incubator 37oC, 10% CO2 in the ImageXpress Room. |
| 6. |  | Wash Wellmate tubing with 4X ddH2O, 4X EtOH, 2X ddH2O. |

### **Day 4-5:** Fluorescent Imaging Protocol. Date:\_\_\_\_\_\_\_\_\_\_ Start Time:\_\_\_\_\_\_\_\_\_\_

### After 1 day incubation at 37oC, 10% CO2, 10 L of 50 M Calcein, AM will be added. Then the plates will be place in the Thermo Incubator in the IXMicro room and the CRS Robot will load and image the plates on the ImageXpress Micro using the protocol below.

|  |  |  |
| --- | --- | --- |
| Task Number | Check List | Action |
| 1. |  | Add 10 L of 50 M Calcein, AM using the Multidrop or Wellmate. (Wash 4X ddH2O, 4X EtOH, 2X ddH2O). |
| 2. |  | Place Plates to the Thermo Cytomat 2C Position 1.1. Put in sequence and use stack 2 if necessary. Plates should be facing in, so barcode/A1 is away from the user. |
| 3. |  | Turn on IXMicro components 1-4. On IXMico computer: Open **MetaXpress 5.3 Fixed Cell** profileand open acquisition protocol **BS\_phrodo\_calAM.HTS** (located in the C:\Users Folder\Spark) |
| 4. |  | Select **Screening** and then Select **Enable External Control** |
| 5. |  | Open Stage and remove plate if one is present. Then close the stage and reopen and close again to reset the plate holder. |
| 6. |  | Make sure the **FITC-FIXED** cube is in position 4 in the IXMicro. |
| 7. |  | Make sure the **TRITC-FIXED** cube is in position 5 in the IXMicro. |
| 8. |  | On Polara Computer: Open **Polara** Software and open **LOR13545.mdr** workspace. |
| 9. |  | Make sure the **Vacuum de-lidding pump is Plugged In**. |
| 10. |  | In JJWu Profile open **IXMCyt Schedule** and select Number of Samples (= # of plates). |
| 11. |  | Click on **Schedule** Button and then select **Run** button. Then Select **Start Run**. Wait until all components are initialized. |
| 12. |  | Then Click the **Play** button to begin the run. |
| 13. |  | When protocol is complete remove plates from Incubator. Turn off IXMicro if no one is signed up to use afterwards. |

Fluorescent Imaging Screening Protocol

|  |  |
| --- | --- |
| FL | Anal. # |
| SP\_01 |  |
| SP\_02 |  |
| SP\_03 |  |
| SP\_04 |  |
| SP\_05 |  |
| SP\_06 |  |
| SP\_07 |  |
| SP\_08 |  |
| SP\_09 |  |
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| SP\_36 |  |
| SP\_37 |  |
| SP\_38 |  |
| SP\_39 |  |
| SP\_40 |  |
| SP\_41 |  |
| SP\_42 |  |

1. Experiment

Settings: Load settings→”from file”→ok→Users folder, Spark, “**BS\_phrodo\_calAM.HTS**”→Open→Next

* 1. Names and Descriptions: **BS-Phrodo-calceinAM**
     1. Experiement Set: **SPARK**
     2. Database: **G28L9Q1**
  2. Objectives and Camera: 4X S Fluor
  3. Plate: Greiner 384-well (16x24) 37C – 100608
     1. Well diameter: 3300 um; Column offset: 12130 um; Row offset: 8990 um; Column spacing: 4500 um; Row spacing: 4500 um; Well depth: 11500 um; Plate length: 127.8 mm; Plate width: 85.5 mm; Plate height: 14.4 mm
     2. Wells to visit: 384 wells of 384
     3. Sites to visit: 1 site/well

1. Time lapse: 1 point
2. Acquisition Loop: # of wavelengths = 2; enable laser and image based focusing
   * 1. Autofocus:
        1. Focus on plate bottom, then offset by bottom thickness.
        2. Image-based focusing: algorithm **Standard**; bin 1; custom exposure times=**ON**; allow image-based focusing for recovery from laser-based well bottom failures=OFF
     2. Initial well for finding sample: Specific Well:**C3**
     3. W1: FITC-FIXED
        1. Exposure: **600 ms**
        2. Auto Expose: target maximum intensity **5000**
        3. Autofocus: Laser and Image-based
           1. Offset (um) from W1: **25**
           2. Image-based range +/1 (um): **100**
           3. Max. step (um): **20**
           4. Exposure (ms): **200**
           5. Gain: **Gain 2 (4x)**
     4. W1: TRITC-FIXED
        1. Exposure: **600 ms**
        2. Auto Expose: target maximum intensity **5000**
        3. Autofocus Lasers with z-offset from W1
           1. Post-laser offset: -**0.02** um
3. Journals- None
4. Display Settings
   * 1. Manually set image display properties
     2. Display images during autofocus
     3. Display images during acquisition
5. Post-acquisition-N/A
6. Summary-N/A

### Fluorescent Image Data Analysis using MetaXpress. Date:\_\_\_\_\_\_\_\_\_\_\_\_\_

1. Follow protocol as indicated in MetaXpress Analysis <**Cell Scoring**> or <**Granularity**>
2. Settings “**Astrocytes-Spark-FITC-TRITC**”
   1. All nuclei: W1 Source: FITC-FIXED
      1. Display result image: Segmentation
      2. Min width: 29 um, Max width: 400 um
      3. Intensity above local background: 30
   2. Positive Marker: W2 Source: TRITC-FIXED
      1. Stained area: Nucleus
      2. Min width: 10 um, Max width: 20 um
      3. Intensity above local background: 30
   3. OR Granules: Granule image: TRITC-FIXED
      1. Algorithm: Standard
      2. Min width: 4 um, Max width: 20 um
      3. Intensity above local background: 70
   4. Nuclear stain: Nuclear image: FITC-FIXED
      1. Min width: 10 um, Max width: 100 um
      2. Intensity above local background: 20
   5. Run Analysis-
      1. Go to Plate Data Utilities, then Run Analysis
      2. Select the plates to run the analysis against.
      3. Select Analysis <**Cell Scoring**> or <**Granularity**>
      4. Select Settings “**Astrocytes-Spark-FITC-TRITC**”
      5. Click OK to run analyses.
3. Export Data
   1. Go to Plate Data Utilities, then Export Measurements
   2. Select Image Measurements, Select plate or plates to export , Click Next, Click Finish
   3. Select “Well Name-Image Measurements” and all “Available Measurement Types”, Click OK
   4. Change Destination Folder to //SPARK, Enter Plate Barcode as Filename (or just use default and write down the matching plate information and data file name above, Check Export Tab Delimited, Click OK, Click Close.
   5. If WellName doesn’t appear in exported file select Cell and Image Measurements in b. above and then delete repeated columns in exported file.

### Analyze Data using Assay Explorer: Protocol “**Spark\_HCS**” or “**Spark\_HCS\_qHTS**”.