FINAL SCRIPT: APPROVED FOR FILMING

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Title: Advanced Glycation End Products Sensitize Human Sensory-Like Neuron Cells to Capsaicin-Induced Calcium Influx

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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?

   A: Yes

   A: We are able to record movies.

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

   A: Yes

3. **Filming location:** Will the filming need to take place in multiple locations?

   A: No

Current Protocol Length
Number of Steps: 18
Number of Shots: 30

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

REQUIRED:

1.1. **Gessica S. A Silva:** Increased collagen-derived advanced glycation end products, or AGEs, are consistently linked to painful diseases. This research investigates whether the glycation process sensitizes sensory neurons to capsaicin excitation.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 3.*

What significant findings have you established in your field?

1.2. **Michelle C. Bufalo:** Our results show that collagen glycation increases calcium influx compared to cells treated with normal collagen, suggesting that sensory-like neurons express functional TRPV1 channels and that glycation increases capsaicin excitation.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 5B, 5C, 5D, Figure 4.*

How will your findings advance research in your field?

1.3. **Marcelo M. de Souza:** This research demonstrates that mimicking a pro-nociceptive environment with glycated collagen leads to up-regulation of capsaicin-induced calcium influx, confirming the functionality of TRPV1 channels and suggesting that AGEs may sensitize these channels.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: LAB MEDIA: Figure 4.*

What research questions will your laboratory focus on in the future?

1.4. **Michelle Cristiane Bufalo:** Since glycation of collagen occurs with aging and in pathological conditions, this approach may serve as a valuable tool for investigating new molecular targets for treating various degenerative diseases. NOTE: Michelle Cristiane Bufalo delivered this statement.

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

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Calcium Influx Imaging

Demonstrator: Marcelo Medina and Michelle Cristiane Bufalo

2.1. To begin, prepare 1 milliliter of Fluo-8 dye-loading solution by adding 2 microliters of Fluo-8 stock solution to 998 microliters of assay buffer **[1-TXT]**.

2.1.1. Talent pipetting 2 microliters of Fluo-8 stock solution into a tube containing 998 microliters of assay buffer. **TXT: Assay buffer: HBSS buffer and 10x Pluronic F127 Plus.**

2.2. Pipette 250 microliters of this solution into each plated dish containing sensory-like neuron cells that are previously differentiated and treated with glycated or non-glycated extracellular collagen matrix **[1]**. Incubate for 30 minutes in a humidified atmosphere of 5% carbon dioxide at 37 degrees Celsius **[2]**.

2.2.1. Talent pipetting 250 microliters of prepared Fluo-8 dye-loading solution into a plated dish with sensory-like neuron cells.

2.2.2. Talent placing the dish inside the incubator.

2.3. After 30 minutes, remove the dish from the incubator **[1]** and keep it at room temperature in the dark for another 30 minutes **[2]**.

2.3.1. Talent removing the dish from the incubator.

2.3.2. Talent placing the dish on the lab bench in a dark area.

2.4. For calcium influx imaging, prepare a syringe with 250 microliters of 2-micromolar capsaicin solution **[1]**. Connect the syringe to a sterile 23-gauge Butterfly scalp vein set **[2]**. NOTE: The VO has been edited.

2.4.1. Talent filling a syringe with 250 microliters of 2 micromolar capsaicin solution.

2.4.2. Talent attaching the syringe to a Butterfly type 23 gauge scalp vein set.

~~2.4.3. Talent securing the syringe onto a lab stand using a clamp.~~ NOTE: This shot was not filmed.

2.5. With extreme caution, place the scalp needle into the Petri dish positioned on the confocal microscope stage without touching the bottom **[1]**. NOTE: The VO has been edited.

2.5.1. ~~Talent placing the Petri dish on the microscope stage.~~ NOTE: This shot was not filmed.

2.5.2. SCOPE: Talent carefully positioning the scalp needle into the Petri dish without making contact with the bottom. NOTE: The videographer filmed this shot.

2.6. Identify a field containing more than 20 cells **[1]** and adjust the focus using bright field light **[1]**.

2.6.1. SCREEN: 68036_Screenshot_2.6.1 and 2.6.2: 00:01-00:18

2.6.2. SCREEN: 68036_Screenshot_2.6.1 and 2.6.2: 00:25-00:33

2.7. Start **Live** mode with a 488-nanometer laser **[1]** and adjust the focus and illumination parameters **[2]**.

2.7.1. SCREEN: 68036_Screenshot_1.mp4 00:03-00:10.

2.7.2. SCREEN: 68036_Screenshot_1.mp4 00:11-00:20.

2.8. Start the acquisition recording **[1]**. After the baseline period of t = 0 to t = 60 seconds, gently push the syringe plunger to release 250 microliters of capsaicin into the Petri dish **[2]**. NOTE: The VO has been edited.

2.8.1. SCREEN: 68036_Screenshot_1.mp4 00:21-00:30.

~~2.8.2. Talent applying 250 microliters of 2-micromolar capsaicin.~~ NOTE: This shot was not filmed, because item 2.8.2 is similar to the item 2.8.3.

2.8.3. Talent pushing the syringe plunger gently to release 250 microliters of capsaicin into the Petri dish. NOTE: This shot was modified during the shoot.

3. Post-Processing and Data Analysis

3.1. Open the microscopy software and select **Quantification** mode to perform the cell analysis **[1]**. Analyze fluorescence spike cells before and after the capsaicin stimulus **[2]**.

3.1.1. SCREEN: 68036_Screenshot_2.mp4 00:03-00:05.

3.1.2. SCREEN: 68036_Screenshot_2.mp4 00:06-00:09.

3.2. Create a Region of Interest or ROI *(R-O-I)* in each responsive cell, ensuring that the entire cell area is included **[1-TXT]**.

3.2.1. SCREEN: 68036_Screenshot_2.mp4 00:15-00:29, 00:52-01:05. **TXT: Responsive cells: Cells that display a fluorescence spike after capsaicin** *Video Editor: Speed up the video as needed.*

3.3. Export the data as a CSV *(C-S-V)* file for further analysis **[1]**.

3.3.1. SCREEN: 68036_Screenshot_2.mp4 01:10-01:19.

~~3.4. Alternatively, perform the analysis using the freely available FIJI *(Fiji)* software **[1].**~~

~~3.4.1. SCREEN: To be provided by authors: Opening FIJI software and displaying the home interface.~~ NOTE: Removed this as OBS studio window is visible here.

3.5. To perform the analysis with FIJI *(Fiji)* software, open the image files by selecting **File**, followed by **Import, Bio-Formats,** and then choose the relevant file **[1]**.

3.5.1. SCREEN: 68036_Screenshot_3.mp4 00:02-00:16.

3.6. Use the **Magnifying Glass** tool to zoom in **[1]** and the **Freehand Selection** tool to draw an ROI around the responsive cells **[2]**.

3.6.1. SCREEN: 68036_Screenshot_3.mp4 00:17-00:20.

3.6.2. SCREEN: 68036_Screenshot_3.mp4 00:21-00:33.

3.7. Press the **T** key to add it to the **ROI Manager [1-TXT]**. NOTE: The VO has been edited.

3.7.1. SCREEN: 68036_Screenshot_3.mp4 00:33-00:35. **TXT: Repeat this for all cells of interest**

3.8. Then, go to the main menu and choose **Analyze**. Select **Mean Gray Value** and click **OK [1]**. Go to the **ROI Manager** window and select **all ROIs [2]**. NOTE: The VO has been edited.

3.8.1. SCREEN: 68036_Screenshot_3.mp4 00:56-01:08.

3.8.2. SCREEN: 68036_Screenshot_3.mp4 01:08-01:12.

3.9. Select **More**, followed by **Multi Measure**. A new window displaying fluorescence intensity measurements across multiple frames will appear **[1]**. NOTE: The VO has been edited.

3.9.1. SCREEN: 68036_Screenshot_3.mp4 01:13-01:21.

3.10. Navigate to **File**, **Save As,** and export the results as a **CSV file [1]**. NOTE: The VO has been edited.

3.10.1. SCREEN: 68036_Screenshot_3.mp4 01:22-01:42.

3.11. Now, import the CSV file into the Google Sheet **[1]**. Normalize the intensity value for each time point **[2]**. Calculate average intensity as F_o *(F-zero)* for all images captures between 0 to 60 seconds **[3]**. Calculate F(t) *(F-T)*, the average of the maximum fluorescence intensity as observed after the stimulus **[4]**.

3.11.1. SCREEN: 68036_Screenshot_4.mp4 00:05-00:18.

3.11.2. SCREEN: 68036_Screenshot_4.mp4 00:36-01:13. *Video Editor: Speed up the video.*

3.11.3. SCREEN: 68036_Screenshot_4.mp4 01:20-01:34.

3.11.4. SCREEN: 68036_Screenshot_4.mp4 01:48-02:01.

3.12. Calculate the **ΔF/F0** *(Delta F over F-zero)* for each ROI using the given equation **[1,2]**. Now, paste the data in the Graphpad *(Graph-pad)* Prism software and select scatter plot to visualize the data **[3]**.

3.12.1. SCREEN: 68036_Screenshot_4.mp4 03:18-03:40.

3.12.2. TEXT ON PLAIN BACKGROUND: $\Delta F/F_o = (F(t) - F_o) / F_o$

3.12.3. SCREEN: 68036_Screenshot_4.mp4 03:47-03:49, 04:00-04:03, 04:08-04:11, 04:17-04:30, 04:36-04:40. *Video Editor: Too many timestamps are added for this shot to avoid showing the OBS studio window.*

Results

4. Representative Results

4.1. β-III *(beta three)* tubulin expression in undifferentiated and differentiated cells is presented in this figure **[1]**. From the results, it is evident that differentiated cells exhibited neuron-like morphology with elongated neurites **[2]**, while undifferentiated cells retained a rounded shape **[3]**.

4.1.1. LAB MEDIA: Figure 3.

4.1.2. LAB MEDIA: Figure 3. *Video Editor: Highlight the right image.*

4.1.3. LAB MEDIA: Figure 3. *Video Editor: Highlight the left image.*

4.2. Differentiated cells also showed an increased expression of TRPV1 *(T-R-P-V-one)* compared to undifferentiated cells **[1]**.

4.2.1. LAB MEDIA: Figure 4. *Video Editor: Highlight B and the dark green bar in C.*

4.3. Cells treated with glycated collagen exhibited a higher capsaicin-induced calcium influx compared to normal collagen-treated cells **[1]**.

4.3.1. LAB MEDIA: Figure 5B, 5C, 5D. *Video Editor: Highlight the bottom two images in 5B, red graph in 5C, and the longer bar in 5D.*

Pronunciation Guides:

1. Fluo-8

Pronunciation link:
https://www.howtopronounce.com/fluo-8
IPA: /fluː.oʊ eɪt/
Phonetic Spelling: floo-oh eight

2. Pluronic F127

Pronunciation link:
https://www.howtopronounce.com/pluronic
IPA: /plʊˈrɑː.nɪk/
Phonetic Spelling: ploo-rah-nik
Note: "F127" is said as "eff one twenty-seven"

3. Capsaicin

Pronunciation link:
https://www.merriam-webster.com/dictionary/capsaicin

IPA: /ˈkæpˌseɪsɪn/
Phonetic Spelling: kap-say-sin

4. Butterfly (scalp vein set)

Pronunciation link:
https://www.howtopronounce.com/butterfly-needle
IPA: /ˈbʌtərˌflaɪ/
Phonetic Spelling: buh-ter-fly

5. Confocal (Microscope)

Pronunciation link:
https://www.howtopronounce.com/confocal
IPA: /ˌkɑːnˈfoʊkəl/
Phonetic Spelling: kon-foh-kul

6. FIJI (Fiji software)

Pronunciation link:
https://www.howtopronounce.com/fiji
IPA: /ˈfiː.dʒi/
Phonetic Spelling: fee-jee

7. ROI (Region of Interest)

Pronunciation link:
https://www.howtopronounce.com/roi
IPA: /ɑːr.oʊˈaɪ/
Phonetic Spelling: ar-oh-eye

8. $\Delta F/F_0$ (Delta F over F-zero)

Pronunciation link (Delta):
https://www.merriam-webster.com/dictionary/delta
IPA (Δ): /ˈdɛltə/
Phonetic Spelling: del-tuh
"F over F-zero" is pronounced as: **eff over eff zero**

9. TRPV1

Pronunciation link:
https://www.howtopronounce.com/trpv1
IPA: /tiː ɑːr piː viː wʌn/
Phonetic Spelling: tee-ar-pee-vee-one

10. β-III Tubulin

Pronunciation link for "tubulin":
https://www.merriam-webster.com/dictionary/tubulin
IPA: /ˈtuːbjəlɪn/

Phonetic Spelling: too-byuh-lin
"β-III" is pronounced as **beta three**