FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 67831
Scriptwriter Name: Nilesh Kolhe
Project Page Link: https://review.jove.com/account/file-uploader?src=20688503

Title: Assessment of ERK Activity Modulation in Early Zebrafish Noonan Syndrome Models via Live FRET Microscopy and Immunofluorescence

Authors and Affiliations: Giulia Fasano[1], Valeria Bonavolontà[2], Catia Pedalino[2], Martina Venditti[2], Graziamaria Paradisi[2], Stefania Petrini[3], Marco Tartaglia[2], Antonella Lauri[2]

[1]Research laboratories, Ospedale Pediatrico Bambino Gesù, IRCCS
[2]Molecular Genetics and Functional Genomics, Ospedale Pediatrico Bambino Gesù, IRCCS
[3]Microscopy facility, Research laboratories, Ospedale Pediatrico Bambino Gesù, IRCCS

Corresponding Authors:
Antonella Lauri antonella.lauri@opbg.net

Email Addresses for All Authors:
antonella.lauri@opbg.net
giulia.fasano@opbg.net
valeria.bonavolonta@opbg.net
catia.pedalino@opbg.net
martina.venditti@opbg.net
graziamaria.paradisi@opbg.net
stefania.petrini@opbg.net
marco.tartaglia@opbg.net

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? **YES**

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

   If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye**.

   Videographer: Please film the following shots labeled SCOPE using a SCOPE KIT

   SCOPE: 2.1.2, 2.2.3, 2.3.2, 3.2.1, 3.2.2, 3.2.3

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

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, One is on the 3rd floor and the other is on the gound floor (Lab 058)**

Current Protocol Length
Number of Steps: 27
Number of Shots: 59

Introduction

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

INTRODUCTION:

1.1. **Antonella Lauri** : We create zebrafish models of complex developmental diseases, linking patient sequencing to functional genomics for variant interpretation and preclinical drug testing.

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

1.2. **Antonella Lauri** : Despite improved FRET ERK sensors, robust in vivo assays detecting early, subtle pathogenic RAS-MAPK fluctuations with sufficient spatiotemporal sensitivity remain lacking.

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

CONCLUSION:

1.3. **Antonella Lauri** : Our live FRET pipeline in zebrafish enables early, sensitive detection of pathogenic ERK signaling changes and supports rapid variant assessment and MEK-inhibitor testing.

1.3.1. 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.

Ethics Title Card

This research follows the ARRIVE guidelines and has been authorized by the Italian Ministry of Health (Direzione Generale della Sanità Animale e dei Farmaci veterinari – DGSAF)

Protocol

2. Microinjection and Treatment of Zebrafish Embryos for FRET Imaging and MEK Inhibition Assays

Demonstrators: Giulia Fasano, Valeria Bonavolontà

2.1. To begin, place the custom microinjection plate containing molded 2% agarose in E3 medium on a working platform **[1]**. Arrange and align fertilized *Teen* zebrafish eggs in the plate to appropriate lanes to restrict egg movement during microinjection **[2]**.

2.1.1. WIDE: Talent placing microinjection plate with molded 2% agarose on a working platform.

2.1.2. SCOPE: Talent arranging and aligning fertilized *Teen* eggs in the molded microinjection plate.
Videographer: Please film the shots labeled SCOPE using a SCOPE KIT

2.2. Backload the needle with 2 microliters of injection material using a microloader pipette **[1-TXT]**. Adjust the pressure and time settings to calibrate each injection based on the needle and embryo quality on the microinjection device **[2]**. Inject the solution into the one-cell stage of Teen zebrafish embryos **[3]**.

2.2.1. Talent loading the needle with injection material using a microloader pipette. **TXT: Injection material: 30 - 60 pg of capped and polyadenylated mRNA in 0.3x Danieau solution**

2.2.2. Talent adjusting the pressure and time settings on the microinjection device.

2.2.3. SCOPE: Talent injecting the solution into the one-cell stage of a Teen zebrafish embryo.

2.3. Raise microinjected Teen embryos under controlled husbandry conditions **[1-TXT]**. Clean out any eggs that appear cloudy or degenerated within 3 hours of deposition **[2]**.

2.3.1. Talent placing microinjected embryos in an incubation chamber. **TXT: Temperature: 28 °C, Humidity: 70%, Light/Dark cycle: 14/10 h**

2.3.2. SCOPE: Talent removing cloudy or degenerated eggs from the injected embryos.

2.4. At approximately 4 hours post-fertilization, using a standard fluorescence stereomicroscope set to a wavelength range of 465 to 500 nanometers **[1]**, screen the embryos for fluorescence reporter expression. Select Teen-positive fish for FRET *(fret)* imaging and reserve negative siblings for immunohistochemical assessments **[2]**.

2.4.1. SCREEN: 2.4.1-2.4.2.mp4 00:00-00:20

2.4.2. SCREEN: 2.4.1-2.4.2.mp4 00:21-end

2.5. Place embryo pools of equal numbers into different wells of a 6-well plate **[1]**. To start treatment, immerse the embryos in 3 milliliters of E3 medium containing vehicle control as DMSO *(D-M-S-O)* or diluted MEK *(M-E-K)* at the desired concentration **[2]**.

2.5.1. Talent distributing embryos evenly into separate wells of the 6-well plate.

2.5.2. Talent adding E3 medium DMSO or MEK inhibitor into the wells of the 6-well plate.

3. Live Multispectral FRET Imaging of RASopathy Zebrafish Models

Demonstrators: Giulia Fasano, Stefania Petrini

3.1. To begin, melt the 1.5% low-melting agarose aliquot in a thermomixer at 50 degrees Celsius. Once dissolved, reduce the temperature to approximately 30 degrees Celsius **[1-TXT]**.

3.1.1. WIDE: Talent placing the LMA aliquot in a water bath.

TXT: Once dissolved, reduce temperature to 30 °C

3.2. Using thin hair, position a single injected Teen-positive embryo at the center of a 35-millimeter glass bottom dish for live imaging **[1]**. Remove excess E3 medium **[2]** and add a drop of low-melting agarose to immobilize the embryo. Allow the agarose to polymerize at room temperature **[3]**.

3.2.1. SCOPE: Talent positioning the embryo at the center of the dish using a thin hair.

3.2.2. SCOPE: Talent removing excess E3 medium using a pipette.

3.2.3. SCOPE: Talent adding a drop of LMA onto the center of the embryo.

3.3. Turn on the incubator controller at least 1 hour before acquisition and set the temperature to 28 degrees Celsius to maintain embryo health **[1]**. Once the incubator stabilizes, place the imaging dish with the embryo on the sample holder **[2]** and use a 10X dry objective to visualize the sample **[3]**.

3.3.1. Talent turning on the incubator controller and setting the temperature.

3.3.2. Talent placing the imaging dish on the sample holder.

3.3.3. Talent selecting the 10x objective and observing the embryos through the objective.

3.4. Switch on the argon-ion laser and adjust the **laser power** to **50%**. In **Hardware Settings**, choose **8-bit depth resolution** to acquire images **[1]**.

3.4.1. SCREEN:.4-3.7.mp4 00:00-00:08

3.5. Next, in the **acquisition panel**, select the **spectral detection *XYλZ* *(X-Y-gamma-lambda-zed)* scanning mode** and set the **format image** to **512 x 512 px** *(pixels)*; **scanning speed** of **400 Hz** *(hertz)* and **optical zoom** of **0.75 [1]**. Activate the **458 nm** *(nanometer)* **laser line** of the **argon-ion laser** and set its **intensity** value to **8.5% [2]**. Then, select a **HyD** *(hybrid)* **detector** and set its sensitivity to a **gain** value of **500 [3]**.

3.5.1. SCREEN: 3.4-3.7.mp4 00:09-00:26
3.5.2. SCREEN: 3.4-3.7.mp4 00:27-00:35
3.5.3. SCREEN: 3.4-3.7.mp4 00:36-00:40

3.6. Open the dropdown menu in the **detector** bar to select the **cyan fluorescence protein** or **ECFP** *(E-C-F-P)* **emission curve [1]**. To display the **Ypet** *(Y-P-E-T)* emission curve, activate a second detector, then select the **yellow fluorescence protein YFP** *(Y-F-P)* **emission curve [2]**.

3.6.1. SCREEN: 3.4-3.7.mp4 00:41-00:47

3.6.2. SCREEN: 3.4-3.7.mp4. 00:48-00:57

3.7. To start live acquisitions, position the detection cursor in the range of the most intense YFP signal to visualize the sample. Set the start and end positions of the sample thickness in the z-stack LAS *(L-A-S)* X window **[1]**. Set the λ *(gamma)*-**scan range properties** to **begin** at **460 nm** and **end** at **570 nm** of the detection range. Define the **Detection Band Width** as 5 nm and **l-scan Stepsize** as **5 nm**. Start z-stack acquisition **[2]**.

3.7.1. SCREEN: 3.4-3.7.mp4 00:58-01:34

3.7.2. SCREEN: 3.4-3.7.mp4 01:44-02:02

4. Postprocessing and Dye Separation for Ratiometric FRET Imaging

4.1. Insert and save two adapted reference emission spectra for CFP and YFP in the dye database available in the **Configuration** window to exclude spectral bleed-through **[1].**

4.1.1. SCREEN: 3.8-3.9.mp4 00:12-00:49

4.2. Select the resulting spectral image file from the imaging session and open the **Process** window **[1]**. Next, in the **Dye Separation tool,** select **Spectral Dye Separation** and configure the settings for the dye separation. In the dropdown lists on the left side of the dialog, select the new CFP emission spectrum in the first position and the new YFP emission spectrum from the spectrum database **[2]**.

4.2.1. SCREEN: 3.8-3.9.mp4 00:50-01:03
4.2.2. SCREEN: 3.8-3.9.mp4 01:29-01:45

4.3. In the **λ-scan** of the images, identify the image with the greatest signal intensity then, move along the Z scan to select the optical section that highlights the area of interest on the margin zone **[1]**.
4.3.1. SCREEN: 3.10-3.13.mp4 00:0-00:16

4.4. Activate the **ROI** *(R-O-eye)* **selection mode** on the margin zone of the animal pole to define the area with the best spectrum **[1]**. Click on **ROICrosshair** in the display window and adjust the size of the reference ROI to **40 voxels** in the **Measurements Area [2-TXT]**.
4.4.1. SCREEN: 3.10-3.13.mp4 00:17-00:27
4.4.2. SCREEN: 3.10-3.13.mp4 00:28-00:40
TXT: Normalize data

4.5. Open the newly generated file containing the two separated channels. Produce a two-dimensional projection image from the three-dimensional image series using the maximum intensity projection **[1]**. In the **Process** window, select **Crop** to separate channels into two separate files, the CH1 *(channel-one)* and CH2 *(channel-one)* channels **[2]**.
4.5.1. SCREEN: 3.10-3.13.mp4 00:41-00:54
4.5.2. SCREEN: 3.10-3.13.mp4 00:57-01:20

4.6. In the **Process** window, select **Combine Images** then select the **CH2 file** and insert it in the **first option**. Then, select the **CH1 file** and insert it in the **second option [1]**. Set a **rescale** with **factor 5** and choose the **Ratio operation [2]**. Click on **Apply** to generate the new file containing the ratiometric image. Save the file for data analysis **[3]**.
4.6.1. SCREEN: 3.10-3.13.mp4 02:00-02:18
4.6.2. SCREEN: 3.10-3.13.mp4 02:19-02:26
4.6.3. SCREEN: 3.10-3.13.mp4 02:27-02:40

5. Quantitative Analysis of FRET Signals and Image Rendering

5.1. Import the spectral imaging and dye separation image files into the open-source Fiji *(fiji)* software for analysis **[1]**.
5.1.1. SCREEN: 3.14.1---3.17.2.mp4 00:00-00:13

5.2. Configure parameters for readout measurements in Fiji using **Analyze** and **Set Measurements**. Select **Area, Integrated Density**, and **Mean Grey Value** as the parameters of interest **[1]**. Using the **polygon selection tool** from the toolbar, select the **region of interest** corresponding to the **margin of the gastrula [2]**. Sequentially, click **Analyze, Tools,** and **ROI Manager** to save the **ROI *x,y* specifications [3].**
5.2.1. SCREEN: 3.14.1---3.17.2.mp4 02:06-02:22
5.2.2. SCREEN: 3.14.1---3.17.2.mp4 02:24-02:36
5.2.3. SCREEN: 3.14.1---3.17.2.mp4 02:37-02:49

5.3. To perform measurements in a selected ROI, click on **Analyze** and **Measure [1]**. Organize FRET by CFP ratio values for each ROI in a worksheet with experimental groups in columns and raw values in rows **[2]**.
5.3.1. SCREEN: 3.14.1---3.17.2.mp4 02:50-02:59
5.3.2. SCREEN: 3.14.1---3.17.2.mp4 04:09-04:28

5.4. For a single biological replicate, create a column table with one grouping variable, where each group is defined by a column **[1-TXT]**.
5.4.1. SCREEN: 3.14.1---3.17.2.mp4 05:00-05:16
TXT: For multiple replicates, create grouped tables with 2 grouping variables

6. Assessment of Morphological Phenotypes and Image Analysis in Zebrafish Embryos

Demonstrator: Valeria Bonavolontà

6.1. At the end of gastrulation, fix the embryos through immersion in 4% paraformaldehyde prepared in PBS for 20 minutes at room temperature **[1]**. After fixation, wash the embryos several times with PBS **[2]**.

6.1.1. WIDE: Talent adding PFA from the labeled container onto the embryos.

6.1.2. Talent transferring PBS from the labeled container onto the embryos.

6.2. Using a Pasteur pipette, orient the embryos laterally in a single well of a 12-well plate containing fresh PBS **[1]**.

6.2.1. Talent using a Pasteur pipette to carefully orient embryos in the 12-well plate.

6.3. Using a stereo microscope with a **2.7X magnification objective** with **0.63X scanning objective and 8.6 zoom factor [1]**, capture the images in a **brightfield mode** to evaluate the presence of an oval shape **[2]**.
6.3.1. SCREEN: 4.3.1-4.3.2.mp4 00:00-00:11
6.3.2. SCREEN: 4.3.1-4.3.2.mp4 00:12-00:39

6.4. Import the captured embryo image into Fiji *(fiji)* software **[1]**. To measure the embryos' axes length, select the **straight tool** from the toolbar and click on **Analyze,** followed by **Measure [2].** After performing selected measurements, add them to the ROI *(R-O-e)* manager list and save the ROI file **[3]**.

6.4.1. SCREEN: 4.4.1-4.5.2.mp4 00:00-00:06

6.4.2. SCREEN: 4.4.1-4.5.2.mp4 00:07-00:16

6.4.3. SCREEN: 4.4.1-4.5.2.mp4 00:17-00:23, 02:57-03:20

6.5. Export the data into a worksheet. Calculate the **axes ratio** by dividing the length of the major axis by the length of the minor axis **[1]**. Calculate the mean, the standard deviation of the mean, or the standard error of the mean of different replicates **[2]**.

6.5.1. SCREEN: 4.4.1-4.5.2.mp4 03:20-03:42

6.5.2. SCREEN: 4.4.1-4.5.2.mp4 03:48-04:13

- **Microinjection**

Pronunciation link: https://www.merriam-webster.com/dictionary/microinjection
IPA: /ˌmaɪkroʊɪnˈdʒɛkʃən/
Phonetic spelling: my-kroh-in-jek-shuhn

- **Agarose**

Pronunciation link: https://www.merriam-webster.com/dictionary/agarose merriam-webster.com
IPA: /əˈgɛɹoʊs/ or /əˈgɑːroʊs/
Phonetic spelling: uh-ga-rose

- **Zebrafish**

Pronunciation link: https://www.merriam-webster.com/dictionary/zebrafish
IPA: /ˈziːbrəˌfɪʃ/
Phonetic spelling: zee-bruh-fish

- **Pipette**

Pronunciation link: https://www.merriam-webster.com/dictionary/pipette
IPA: /pɪˈpɛt/
Phonetic spelling: pi-pet

- **Embryo**

Pronunciation link: https://www.merriam-webster.com/dictionary/embryo
IPA: /ˈɛmbrioʊ/
Phonetic spelling: em-bree-oh

- **Husbandry**

Pronunciation link: https://www.merriam-webster.com/dictionary/husbandry
IPA: /ˈhʌz.bən.dri/
Phonetic spelling: huz-buhn-dree

- **Fluorescence**
Pronunciation link: https://www.merriam-webster.com/dictionary/fluorescence merriam-webster.com+1
IPA: /flɔˈrɛsəns/
Phonetic spelling: fluh-res-uhns

- **Stereomicroscope**
Pronunciation link: https://www.merriam-webster.com/dictionary/stereomicroscope merriam-webster.com
IPA: /ˌstɛri.oʊˈmaɪkrəˌskoʊp/
Phonetic spelling: ste-ree-oh-my-kroh-skohp

- **Nanometer**
Pronunciation link: https://www.merriam-webster.com/dictionary/nanometer
IPA: /ˈnænəˌmiːtər/
Phonetic spelling: nan-oh-mee-ter

- **Immunohistochemical**
Pronunciation link: https://www.howtopronounce.com/immunohistochemical
IPA: /ˌɪmjuˌnoʊ-hɪstəˈkɛmɪkəl/
Phonetic spelling: im-yoo-no-his-tuh-kem-i-kul

- **Multispectral**
Pronunciation link: https://www.merriam-webster.com/dictionary/multispectral
IPA: /ˌmʌltiˈspɛktrəl/
Phonetic spelling: mul-tee-spek-truhl

- **Thermomixer**
Pronunciation link: No confirmed link found — not listed in major dictionaries under that exact compound.
IPA (approximate): /ˈθɜrmoʊˌmɪksər/
Phonetic spelling: ther-mo-mix-er

- **Objective** (as in microscope objective)
Pronunciation link: https://www.merriam-webster.com/dictionary/objective
IPA: /əbˈdʒɛktɪv/
Phonetic spelling: uh-jek-tiv

- **Argon** (as in argon-ion laser)
Pronunciation link: https://www.merriam-webster.com/dictionary/argon
IPA: /ˈɑrgɑn/ or /ˈɑrgən/
Phonetic spelling: ar-gon

- **Spectral**

Pronunciation link: https://www.merriam-webster.com/dictionary/spectral
IPA: /ˈspɛktrəl/
Phonetic spelling: spek-truhl

- **Ratiometric**

Pronunciation link: https://www.howtopronounce.com/ratiometric
IPA: /ˌreɪʃiəˈmɛtrɪk/
Phonetic spelling: ray-shee-oh-meh-trik

- **Paraformaldehyde**

Pronunciation link: https://www.merriam-webster.com/dictionary/paraformaldehyde merriam-webster.com
IPA: /ˌpɛrəˌfɔrˈmældəˌhaɪd/
Phonetic spelling: pa-ruh-for-mal-dahy-hyde

- **Pasteur** (as in Pasteur pipette)

Pronunciation link: https://www.merriam-webster.com/dictionary/pasteur
IPA: /pæsˈtɜr/ or /pɑːˈstɜr/ depending on accent — in American often "pas-TER."
Phonetic spelling: pas-ter

- **Brightfield** (microscopy)

Pronunciation link: https://www.merriam-webster.com/dictionary/brightfield
IPA: /ˈbraɪtˌfiːld/
Phonetic spelling: brite-feeld

- **Gastrulation**

Pronunciation link: https://www.merriam-webster.com/dictionary/gastrulation
IPA: /ˌgæs.truːˈleɪʃən/
Phonetic spelling: gas-tru-LAY-shuhn