

Aug 14, 2025

Semi-Automated Analysis Pipeline for Synaptic Function Studies Using pHluorin Biosensors

 In 1 collection

DOI

<https://dx.doi.org/10.17504/protocols.io.kqdg31931l25/v1>

Camila Pulido^{1,2}, Tim Ryan^{1,2}

¹Department of Biochemistry, Weill Cornell Medicine, New York, NY 10065, USA;

²Aligning Science Across Parkinson's (ASAP) Collaborative Research Network, Chevy Chase, Maryland 20815, USA



Camila Pulido

Weill Cornell Medicine

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.kqdg31931l25/v1>

Protocol Citation: Camila Pulido, Tim Ryan 2025. Semi-Automated Analysis Pipeline for Synaptic Function Studies Using pHluorin Biosensors. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.kqdg31931l25/v1>



License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: August 09, 2025

Last Modified: August 14, 2025

Protocol Integer ID: 224344

Keywords: ASAPCRN, pHluorin, Image Processing, Neurons, Data Analysis, synaptic activity within synaptic bouton, synaptic bouton, synaptic function study, engineered phluorin biosensor, phluorin biosensors this protocol, phluorin biosensor, synaptic activity, using phluorin biosensor, analysis for the detection

Funders Acknowledgements:

ASAP

Grant ID: 000580

Abstract

This protocol delineates the analysis for the detection of synaptic activity within synaptic boutons, employing the genetically engineered pHluorin biosensors.

Materials

This protocol is intended to use two analysis programs: [ImageJ \(Fiji\)](#) and [IGOR Pro \(wavemetrics\)](#).

Before start

- This protocol is intended to use two analysis programs: [ImageJ \(Fiji\)](#) and [IGOR Pro \(wavemetrics\)](#).
- Github scripts repository is [here](#).
- Prior to implementing this protocol, users need to customize the provided code to align with their individual settings.
- Maintain consistent file naming formatting across all experiments.
- Each "...50AP" file corresponds to a stack of 250 frames acquired at 4Hz with 0.1s frame exposure. Electrical stimulation (1 train of 50APs at 10Hz) was triggered at frame 50. The total number of "...50 AP" files vary across experiments and its indeed relevant information for the understanding of neuronal function.
- "NH4Cl" file corresponds to a stack of ~ 50 – 100 frames acquired at 2Hz with 0.1s frame exposure. NH4Cl was pipetted in the neuronal chamber and a stable fluorescence signal increase is recorded.

Image processing – synaptic boutons raw signal extraction:

- 1 Synaptic activity can be detected by the increase in fluorescent signal within boutons during electrical stimulation timelapse.
- 2 Signal information corresponding to that specific bouton can be extracted and save by using ImageJ software and 'Time Series Analyzer'
- 3 Drag into ImageJ a control image stack file (i.e Glucose 5 mM).
- 4 Run 'pHluorin_DeltaPeak' macro to get pixel increase signals that correspond to electrical activity in control.
- 5 Place round ROIs by clicking in all synaptic boutons which fluorescent signal increase during electrical stimulation.
- 6 Fill the input variables in 'pHluorin_RawDataExtraction' routine file, with the specifics of your experiment: Path in and out, date, conditions and number of repetitions per condition.
- 7 Having ROIs chosen, semiautomatically extract and save signal information from every frame, looping across all stack files for every condition (i.e. Glucose 5mM, Glucose 0mM, NH4Cl, pharmacology, protein KD, etc.) in one neuron (or experiment), by simply executing the 'pHluorin_RawDataExtraction'.
- 8 The cleaned data outputs are saved in the desired path. Consisting of xls files with each ROI signal and txt files of the average signal of all ROIs per processed stack at every experimental condition and repetition.
- 9 Ensure to save the selected ROIs for future reference (last steps in the code).

Image processing – synaptic boutons background signal extraction:

- 10 Drag into ImageJ a control image stack file (i.e Glucose 5 mM).
- 11 Select “freehand selections” from ImageJ tools bar.



- 12 Draw background ROIs surrounding the synaptic boutons of interest.
- 13 Fill the input variables in 'pHluorin_BGDataExtraction' routine file, with the specifics of your experiment: In and out directory path, date.
- 14 Execute 'pHluorin_BGDataExtraction' script.
- 15 The cleaned background data outputs are saved in the desired path. Consisting of txt files of the average signal of all ROIs per processed stack at every experimental condition and repetition.

pHluorin Synaptic Signal Analysis:

- 16 Open IGOR-PRO program.
- 17 Run the 'pHluorin_LoadRawData()' function with the corresponding variable inputs specific to your experiment to import the signal information from txt files containing the average signal of all ROIs, along with their corresponding background signals, organizing them into wave arrays for further analysis.
- 18 This routine automatically executes all analytical script steps to extract relevant information. The signal data processing steps are as follows:
 - 18.1 Signal of each data point is background corrected by subtracting to the raw signal their corresponding background noise at every. As an output, raw signals are now F corrected signal.
 - 18.2 Next, the script calculates the ΔF values, by subtracting the initial fluorescence values before the action potential train from all values, so that the first produced values will now be around 0.
 - 18.3 Next, the preprocessed data can be normalized to the total sensor expression, making possible to compare signals across experiments. pHluorin biosensor expression is extracted as the maximum signal in the $\Delta F_{\text{NH}_4\text{Cl}}$ wave. Dividing all fluorescence values with the calculated $\Delta F_{\text{NH}_4\text{Cl}}$, will yield the $\Delta F_{\text{APs}}/\Delta F_{\text{NH}_4\text{Cl}}$ normalization of the dataset.
 - 18.4 The dataset is further normalized internally to the fluorescence peak during the AP train. This allows to specifically look at synaptic vesicle endocytic kinetics and is unaffected by changes in exocytosis. First, calculate the ΔF_{MAX} by identifying the maximal fluorescence values around the end of the action potential train. For 10 Hz trains, we

locate the F_{MAX} at a 2 second window around the end of the action potential train.

Dividing all fluorescence values with the calculated ΔF_{MAX} , will yield the $\Delta F_{APs}/\Delta F_{MAX}$ normalization of the dataset.

- 18.5 Finally, the "Endocytic Block" parameter per experiment is calculated as the AP train round number where the $\Delta F_{APs}/\Delta F_{MAX}$ exceeds 50% of normalized signal, measured at 3τ seconds after AP train. 3τ time is initially calculated as fixed variable from the control dataset.
- 18.6 All produced time trace data and subsequent quantifications can be plotted and visualized using IGOR-Pro graph and layout tools.