August 8th, 2014

Object: Rebuttal Letter,

Please find enclosed a point-to-point answer to reviewer’s comments.

We would like to thank all reviewers for helpful suggestions.

**Reviewers' comments:**

**Reviewer #1:**

*Manuscript Summary:*

Authors used combination of pH sensitive DNA encoded sensors and TIRFM imaging technique to explore vesicle recycling events in vivo. Authors recorded imaging in two conditions; vesicle dynamics under resting as well as stimulated conditions. Authors found that as expected more florescence intensity in stimulated condition compared to resting suggesting that more vesicles fused with the plasma membrane. Authors did a great job by using similar cells for resting and stimulated conditions. Authors also described methods and results sections well. However, I would like to mention some comments here.

*Major Concerns:*

1. I do not think authors developed this technique and it was published before. I would like to ask authors what is the new concept here in this paper. Authors should explain what is the difference between their method compared to previous published method. The modifications should be reported in this paper.

**Editor’s Note:** We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

**Author’s Note:** the TIRFM approach is not novel but few works directly address the mechanisms of vesicle fusion and recycling with this technique in primary neuronal cultures. This is because TIRFM requires cells which grow adherent to a glass cover and are sufficiently flat to allow stable visualization of membranes and fusion events.

We demonstrate for the first time that the SH-SY5Y human neuroblastoma cell line can be a valuable model to investigate neurotransmitter release under resting and stimulated conditions, with the TIRFM approach.

We provide a protocol for growing these cells adherent to the glass cover, which is paramount for TIRFM.

Finally, we describe a semiautomatic procedure, developed in the laboratory, for image processing and data analysis, at whole-cell and single-event levels.

The efficacy of the protocols is highlighted by results presented which demonstrate that potassium depolarization induces a massive release of neurotransmitter (known) and changes the behavior/kinetics of fusion events (new). The stimulus-dependent modification of fusion mechanisms is an emerging idea and has been recently found also in other secretory cell types by TIRFM (synaptic-like microvesicles exocytosis in endocrine cells of the pancreas. Bergeron t al, 2014)

2. Did authors use any drugs which inhibit or delay exocytosis events and see the fluorescence intensity decreases with inhibitors as shown KCl increased fluorescence intensity.

The imaging approach and data analysis were developed in our lab in order to verify the impact of newly identified vesicle proteins and their pathological mutants on neurotransmitter exocytosis.

Using different constructs and the described methods, we were able to measure both an increase and a decrease of basal synaptic activity.

*Minor Concerns:*

1. authors need to report in the long abstract what they found. Needs to mention results in one or 2 sentences in abstract.

Following reviewer suggestions, the abstract has been modified. Now a consistent part of the abstract is devoted to the developed methods and to results.

2. Although authors explained trouble shooting about morphological changes and fluorophore, authors did not discuss about limitations of method.

A paragraph related to limitations of method has been inserted in the introduction (data analysis) and in the discussion sessions (accurate selection of the cellular model, given that TIRFM can work only with firmly attached cells).

**Reviewer #2:**

The manuscript by Federica et al., provides a rather detailed description of the technique using total internal reflection fluorescence microscopy (TIRFM) and fluorescent protein expression for visualization and quantification of vesicle release in adherent secretory cultured cells. TIRFM and its applications to study vesicle release are not novel and have been amply described in the literature before. However, this paper/video will still benefit a potential novice user if it includes a more thorough yet generalized description of the protocol and likely pitfalls to be encountered.

*Major comments:*

1. The protocol cannot be easily followed. It has too many details describing the TIRF setup, while the algorithms for data analysis are not well presented.

The TIRF setup has been reduced to essential information.

The algorithms for data analysis have been developed, a workflow for image processing and data analysis has now been included in figure 3 (New figure).

a. In the protocol, the section 1.1 is best to be split into two sub-sections, one concerting SH-SY5Y culture maintenance and the other cell coverslip plaiting. Also, please specify the thickness, glass type and diameter of coverlips optimal for TIRFM, any special coverslip pre-treatment (poly-d-lysine, collagen, etc. or none). In all protocol sections, please provide specific estimated volumes of solutions one might need per experiment or a plate of cells instead of saying "opportune volume". Do you maintain cells on glass coverlips or on plastic please make it clear? Do you filter or otherwise sterilize your solutions?

* Section 1.1 has been splitted in two sections: “SH-SY5Y cell culture” and “cell plating for imaging analysis”
* Coverslips characteristics are specified in the text and in the “Table of Materials and Equipment”
* Coverslip pre-treatment has now been specified in the method, no coating material is used.
* All solution volumes are specified through the text.
* The requested details about cell maintenance and sterilization of solution have been added.

b. Section 2 needs to include only essential details about the TIRFM system necessary to interpret the results presented in the manuscript , but still sufficient to replicate the findings in a different TIRF system (illumination source, excitation/emission filters, dichroic mirror, objective lens specs, CCD camera. The sample preparation (2.2) and sample imaging (2.3) sections can be merged and shortened to just essential steps. When is typically pHluorin expression sufficient for imaging?

* Section 2, now includes only essential information about the microscope setup
* Sample preparation and imaging have been merged and shortened.
* A sentence about optimal pHluorin expression for imaging has been added (paragraph 2.2.5)

c. In section 3, what program is used for image analysis, ImageJ? Where does one download it from with the right set of plug-ins? Provide a brief description of the code/algorithm used in the macro. Define roundness score or cite an article where it can be found.

**Editor’s Note:** Please provide download URL's in the Table of Materials and Equipment.

**Author’s comment**:

The code/algorithm has been described.

We are not allowed to use commercial language through the text (journal policy), but a list of softwares employed and corresponding URL’s is reported in the “Table of Materials and Equipment”.

The following programs have been used:

-Image Pro-Plus, Media Cybernetics software for image analysis (spot selection, ROIs selection, fluorescence intensity determination).

- Excell, Microsoft for photobleaching estimation and correction, whole-cell and single-vesicle analyses. The macros have been developed in our lab using logical and mathematical functions already defined in the Excell program.

- Excell/Prism3 for statistical evaluation.

The specific macros developed in the lab are available on request.

A list of equivalent macros, freely available, is reported at the URL:

<http://rsbweb.nih.gov/ij/docs/guide/index.html> (Table of Materials and Equipment).

2. It is known that strong stimuli lead to a calcium-dependent acidification of the cytosol and mirrored alkalization in the extracellular space via PMCA see Rossano et al., 2013. It is thus likely that extracellular pH increases during KCl stimulation, especially in a tiny volume between the cell and the surface of the coverlslip leading to the exaggeration of pHluorin fluorescent signal and the calculated values of evoked vesicle release. This issue needs to be addressed in the discussion. For a more accurate estimate of evoked vesicle release, one would also need to perform measurements of plasma membrane expressed pHluorin fluorescent responses to a given KCl concentration, and use it as an adjustment factor.

This part has been addressed in the discussion (fluorophores lines 643-648).

We always place a “background” ROI on a surface region of the cells without synaptic events. This helps us in monitoring and estimate possible fluorescence variations induced by stimulation.

*Minor fixes:*

Line 57: fluorescent pH-sensitive DNA-encoded sensors

Line 59: with synaptobrevin is expressed in cultured cells to label the lumen of synaptic vesicles

Line 101: to proteins in the vesicle membrane

Line 102: include a sentence on the kinetics of pHluorin responses (faster than a fusion event)

Line 126: TIRFM

Line 128: "The simultaneous recording of pixels" replace with "Chip-based image acquisition"

Line 166: Fix EDTA spelling

Line 203: Petri dish containing coverslips with cells

Line 240: KCl-KRH provide exact concentrations for every component

Line 358/384: "peak width" not "peak wide"

Line 377: "Fluorescent signal from a single fusion event is shown"

Line 389: as during the control recording

Line 398: width not spread?

Line 403: cumulative fluorescence values

Line 422/433: show a square in the image not a circle

Line 469: define "TIRF zone"

Line 476: the evanescent field with the depth of 70-200 nm

Lines 494-505: need to be merged and rewritten more clearly

All minor concerns have been fixed. Thanks.

Line 424/436: You imaged control and KCl experiments under the same conditions - 1Hz acquisition rate. In Fig 3B (70-80 frame) and Fig 4B show only relevant frames (do not show every frame).

The acquisition rate was 1Hz, but only every second frame was shown in the figure 4B (now reported in figure 5A and 5C). A sentence has been added to explain this discrepancy.

Line 514: Please mention that bleaching in many instances follows a simple exponential decay function, which makes it easier to estimate it and correct for it by performing control recordings.

A sentence explaining that bleaching in many instances follows a simple exponential decay function has been added in the discussion (bleaching paragraph).

**Reviewer #3:**

*Manuscript Summary:*

The manuscript attempts a step-by step description of procedures that involve a combination of biophysical and cell biology techniques and allow registration of synaptic vesicles exocytosis using an advanced imaging approach TIRFM in cultured neuroblastoma cells transfected with a genetically encoded optical indicator of vesicle release and recycling pHluorin, as well as data analysis focused on assessment of these processes' dynamics.  
  
*Major Concerns:*

The clarity of presentation is the most problematic part of the submitted manuscript.

Its illustrations do not present sufficient details and explanations that would allow another researcher in the field to easily comprehend and replicate procedures.

As suggested, the illustrations have been modified.

* Figure 1 has been modified, now it includes a schematic view of the TIRF microscope set-up. Our system is shown in the inset.
* Figure 2 has been modified. Now it describes the procedure to achieve TIRF illumination.
* Figure 3. A new figure illustrating the workflow of image processing and analysis has been added.
* Figure 4 has been modified. Now it describes whole-cell analysis in resting and stimulated cells
* Figure 5 has been modified. Now it describes single-vesicle analysis in resting and stimulated cells

Procedure called "2.1) Perform TIRF imaging with a commercial TIRF system" (line 211), and illustrated with a still picture of their microscope, does not quite meet expectations/definition of a single step of multistep description of the process.

The figure 1 has been modified, now it includes a schematic view of the TIRF microscope set-up. Our system is shown in the inset.

Yet, I believe that both technical quality and efficiency of the presentation could be improved by the authors to the extent that would make this article a useful source for other researchers.  
  
*Minor Concerns:*

It would help for the clarity of the report to having a native English speaker proof-reading the article before submission.  
The beginning part of the Abstract(s), as well as a large portion of the Introduction, is devoted to molecular mechanisms of synaptic transmission, leaving less space for introducing the described techniques.  
The first part of abstract and introduction have been modified

Please check terminology used in the paper. E.g., voltage sensitive Ca2+-channels (line 73), isn't an established term. You may use either voltage-gated or voltage-dependent instead.  
This part has been eliminated. The terminology in the paper has been checked.

Reference "9" (lines 101, 108 and 186) does not describe pHluorin-s or discuss their application to study vesicle recycling, but rather introduces TIRF microscopy.

By mistake, in the old version, the reference list was shifted. Now the reference 9 refers to the paper of Miesenböck and coworkers (Nature. 394(6689):192-5, 1998) which describes pHluorin-s and discusses their application to study vesicle recycling.

SH-SY5Y cell culture section does not refer to any previously described procedures, and provides no comment about its possible modification by the authors.

The paper reporting the original description of SH-SY5Y culturing has been included in the text and in the reference list. Modifications introduced by our laboratory have been reported in the protocol.

Instead of using one section describing configuration of the microscope and all settings used for imaging, authors list them as four procedural steps with sentences starting with the same " Use… wording (line 211, 215, 219 and 224).

The section “microscope set up” has been modified and reduced to the essential. It is still listed as more procedural steps because as requested by the Science editor.

Section 2.2) is called "Sample preparation", but it actually talks about "Turning lasers on" (line 230) or "performing imaging" (line 243).

Section 2.2 has been modified, it contains the procedure for getting TIRF illumination and is called “How to achieve TIRF illumination”

This section also suggests "2.2.5) to stabilize the imaging chamber" (line 248), but does not explain how or what does it mean/involve, as well as utilize an obscure term/word "opportune" (line 234).  
After having reached TIRF conditions, leaving the object in contact with the coverslip few minutes before starting TIRFM imaging, reduces the presence of interference fringes, in our experience.  
All terms “opportune” in the text have been substitute with the exact volume of solution.

Line 255 (2.3.1) does not specify meaning of switching to "in live mode". Does it refer to hardware or software setting?

It refers to a software setting. The term has been specified in the text

Line 263 (2.3.2) suggests using "established imaging frequency" without explaining or referring to procedures that establish it.

The imaging frequency has been specified.

Line 280 (3.1.1) would benefit from mentioning software that runs the referred macro.  
We are not allowed to use commercial language through the text (journal Policy). The softwares used have been specified in “Table of Material and equipment”.

-Image Pro-Plus, Media Cybernetics software for image analysis (spot selection, area selection, fluorescence intensity determination).

- Excell,Microsoft for photobleaching correction, whole-cell and single-vesicle analyses. The macros have been developed in our lab using logical and mathematical functions already defined in the Excell program.

- Excell/Prism3 for statistical evaluation.

The URL’s of downloadable macros has been added (3 section)

*Additional Comments to Authors:*

Provided 2 videos do not demonstrate any particular procedures described in the paper, but rather show examples of their recorded data.

It would be of advantage for authors to look at a couple other JoVE papers with videos before submitting their new revised version.

The video showing the procedures described in the paper will be recorded by JoVe, once the paper will be accepted.

With the provided videos, we just meant to give a general demonstration of our data and analysis.

However, following your suggestions, we illustrate the procedures in the new figures.

Listed above drawbacks are just examples of many other instances of confusing description that misses details, references or connections to other parts of the paper.

We did our best to try to fix all drawbacks present in the paper.