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TITLE:

Measuring Membrane Lipid Turnover with the pH-sensitive Fluorescent Lipid Analog ND6

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SUMMARY:

This protocol presents a fluorescence imaging method that uses a class of pH-sensitive lipid fluorophores to monitor lipid membrane trafficking during cell exocytosis and the endocytosis cycle.

ABSTRACT:

Exo-/endocytosis is a common process mediating the exchange of biomolecules between cells and their environment and between different cells. Specialized cells use this process to execute vital body functions such as insulin secretion from β cells and neurotransmitter release from chemical synapses. Owing to its physiological significance, exo-/endocytosis has been one of the most studied topics in cell biology. Many tools have been developed to study this process at the molecular level, because of which much is known about the protein machinery participating in this process. However, very few methods have been developed to measure membrane lipid turnover, which is the physical basis of exo-/endocytosis.

This paper introduces a class of new fluorescent lipid analogs exhibiting pH-dependent fluorescence and demonstrates their use to trace lipid recycling between the plasma membrane and the secretory vesicles. Aided by simple pH manipulations, those analogs also allow the quantification of lipid distribution across the surface and the intracellular membrane compartments, as well as the measurement of lipid turnover rate during exo-/endocytosis. These

novel lipid reporters will be of great interest to various biological research fields such as cell biology and neuroscience.

INTRODUCTION:

The lipid bilayer is one of the most common biomolecule assemblies and is indispensable for all cells. Outside cells, it forms the plasma membrane interfacing cells and their environment; inside cells, it compartmentalizes various organelles specialized for designated functionalities. Rather dynamic than still, lipid membranes constantly experience fusion and fission, which mediates biomaterial transport, organelle reform, morphology change, and cellular communication. Undoubtedly, the lipid membrane is the physical foundation for almost all cellular processes, and its dysfunction plays a crucial role in various disorders ranging from cancer[1] to Alzheimer's disease[2]. Although lipid molecules are far less diverse than proteins, membrane research so far has mainly been protein-centric. For example, a lot more is known about protein machinery than about lipids in exocytosis[3–5]. Moreover, the organization, distribution, dynamics, and homeostasis of lipids across surface and intracellular membranes largely remain unexplored in comparison to membrane proteins[6].

This is not surprising as modern molecular biology techniques, such as mutagenesis, provide a methodological advantage for studying proteins rather than lipids. For example, transgenic tagging of pH-sensitive green fluorescent protein (GFP a.k.a., pHluorin) to vesicular proteins facilitates the quantitative measurement of the amount and rate of vesicular protein turnover during exo-/endocytosis[7–9]. However, it is almost impossible to genetically modify membrane lipids *in vivo*. Moreover, qualitative and even quantitative manipulations of protein amounts and distributions are much more feasible than those of lipids[10]. Nevertheless, native and synthetic fluorescent lipids have been isolated and developed to simulate endogenous membrane lipids *in vitro* and *in vivo*[11]. One group of widely used fluorescent lipids are styryl dyes, e.g., FM1-43, which exhibit membrane-enhanced fluorescence and are a powerful tool in studying synaptic vesicle (SV) release in neurons[12]. Lately, environment-sensitive lipid dyes have been developed and widely used as a new class of reporters to study various cell membrane properties, including membrane potential[11], phase order[13], and secretion[14].

A new class of lipid mimetics whose fluorescence is both pH-sensitive (e.g., pHluorin) and membrane-sensitive (e.g., FM1-43) was developed to directly measure the lipid distribution in the plasma membrane and endosomes/lysosomes and the lipid traffic during exo-/endocytosis. The well-known solvatochromic fluorophores exhibiting push-pull characteristics due to intermolecular charge transfer were selected for this purpose. Among existing solvatochromic fluorophores, the 1,8-naphthalimide (ND) scaffold is relatively easy to modify, versatile for tagging, and is unique in photo-physics[15] and has therefore been used in DNA intercalators, organic light-emitting diodes, and biomolecule sensors[16–18].

Attaching an electron-donating group to the C4 position of the ND scaffold generates a push-pull structure, which leads to an increased dipole moment by redistributing the electron density in the excited state[19,20]. Such an intramolecular charge transfer produces large quantum yields and Stokes shifts, resulting in bright and stable fluorescence[21]. This group has recently developed a

series of solvatochromic lipid analogs based on the ND scaffold and obtained them with good synthetic yields[20].

Spectroscopic characterization shows that among those products, ND6 possesses the best fluorescence properties (**Figure 1**)[20]. First, it has well-separated excitation and emission peaks (i.e., ~400 nm and ~520 nm, respectively, in **Figure 2A,B**) compared to popular fluorophores such as fluorescein isothiocyanate, rhodamine, or GFP, making it spectrally separatable from them and thus useful for multicolor imaging. Second, ND6 exhibits a more than eight-fold increase in its fluorescence in the presence of micelles (**Figure 2C**), suggesting a strong membrane-dependency. Prior live-cell fluorescence imaging studies with different types of cells showed excellent membrane staining by ND6[20]. Third, when solvent pH is decreased from 7.5 (commonly found in extracellular or cytosolic environments) to 5.5 (commonly found in endosomes and lysosomes), ND6 shows an approximately two-fold increase in fluorescence (**Figure 2D**), showing its pH-sensitivity. Moreover, molecular dynamics simulation indicates that ND6 readily integrates into the lipid bilayer with its ND scaffold facing out of the membrane and piperazine residue showing strong interactions with phospholipid head groups (**Figure 3**). Altogether, these features make ND6 an ideal fluorescent lipid analog to visualize and measure membrane lipid turnover during exo-/endocytosis.

This paper presents a method to study the turnover rate and dynamics of SV lipids using cultured mouse hippocampal neurons. By stimulating neurons with high K^+ Tyrode's solutions, SVs and the plasma membrane were loaded with ND6 (**Figure 4A,B**). Subsequently, neurons were re-stimulated with different stimuli followed by NH_4Cl-containing and pH 5.5 Tyrode's solutions (**Figure 4D**). This protocol facilitates the quantitative measurement of the assembled exocytosis and endocytosis rates under different circumstances (**Figure 4C**).

PROTOCOL:

The following protocol includes (1) a simplified procedure for establishing mouse hippocampal and cortical cultures based on a well-established protocol[22], (2) a brief introduction to an epifluorescence microscope setup for live neurons, (3) a detailed description of loading and imaging ND6 in mouse neurons, (4) a discussion about the quantification of membrane trafficking by ND6 signal. All procedures follow the biosafety and animal guidelines of Florida Atlantic University. The synthesis of ND6 has been described previously[20].

1. Preparation of mouse hippocampal and cortical cultures

NOTE: If not specified otherwise, all steps must be performed in a biosafety level 2 laminar flow hood. Sterilize all tools and materials (if not specified otherwise).

1.1. Use size 5 forceps to place glass coverslips in multiwell culture plates.

NOTE: For example, a 24-well plate is ideal for 12 mm ∅ coverslips.

1.2. Add the appropriate volume of extracellular matrices to coat the cell culture surface (e.g., 75 μL of basement membrane matrix solution per 12 mm ∅ coverslip; see the **Table of Materials**)

1.3. Place the prepared plates in the tissue culture incubator (5% CO_2, 100% humidity, and 37 °C) for 1–4 h to allow basement membrane matrix crosslinking.

1.4. Sacrifice the animals, open the skull, and transfer the whole brain to a 35 mm Petri dish with 3 mL of Hank's Balanced Salt Solution (HBSS) containing 20% fetal bovine serum (H+20). Cut the brain along the midline, and separate the cortices and hippocampi in a laminar flow dissection hood to reduce contamination.

1.5. Use microscissors to cut the tissues into small pieces (~1 mm^3). Transfer those tissues to a 15 mL conical tube containing 5 mL of H+20.

1.6. Wash the tissues three times with 5 mL of H+20 and three times with 5 mL of HBSS.

1.7. Add 1.5 mL of 1% trypsin with ethylenediamine tetraacetic acid and incubate at 37 °C for 10 min for enzyme digestion.

1.8. Repeat step 1.6 and use vacuum to aspirate HBSS in the end.

1.9. Dissociate the tissues mechanically in 1 mL of HBSS containing 2.95 g/L $MgSO_4 \bullet 7H_2O$ using a fire-polished glass pipette.

1.10. Centrifuge the cell suspension at 400 × *g*, 4 °C for 5 min.

1.11. Aspirate the supernatant and resuspend the cells in an appropriate volume of culture medium to obtain a concentration of ~10,000,000 cells/ml.

1.12. Plate the cells at ~1,000,000 cells/cm^2 and place the culture in the incubator (5% CO_2, 100% humidity, and 37 °C) for 1–4 h to facilitate cell attachment to the glass coverslips.

1.13. Add an appropriate volume of culture medium (e.g., 1 mL per well for a 24-well plate). Add the same volume of culture medium after 1 week. After 2 weeks, replace half of the existing culture medium with fresh medium every week.

2. Microscope setup for live-cell fluorescence imaging

NOTE: An exemplary imaging setup (**Figure 5**) includes at least an inverted fluorescence microscope (see the **Table of Materials**), fluorescence light source with automatic shutter, fluorescence filter sets (e.g., for imaging ND6, use 405/10 BP for excitation, 495 LP for dichroic, and 510/20 BP for emission), and a high-sensitivity camera (**Table of Materials**), all of which are controlled by image acquisition software (**Table of Materials**).

2.1. Prepare an imaging chamber that allows temperature control and solution input/output for live-cell fluorescence imaging.

NOTE: For example, a modified open-bath imaging chamber fixed on a heating platform was used in this protocol (**Figure 6**).

2.2. Set up a programable device to switch the perfusion solutions and deliver the electric stimulus at defined time points during imaging.

NOTE: Hardware and software synchronization is necessary for quantitative analyses (**Figure 7**). For example, in this protocol, a trigger output from the imaging camera was used to start a computer program that controls an automatic switch device, which controls a multichannel perfusion system and a square-pulse stimulator.

2.3. To co-image two or more fluorescent reporters, perform multichannel fluorescence imaging either by sequentially switching filter sets or by simultaneously splitting different fluorescence emissions and projecting to the same camera.

3. Loading and imaging ND6 in neuronal cultures

3.1. Weigh out an appropriate amount of ND6, dissolve it in dimethylsulfoxide (DMSO), and allow it to solubilize at room temperature; sonicate briefly (e.g., 3 min). Filter the crude stock solution using a 0.22 μm filter to remove large dye aggregates. After filtration, determine the dye concentration by absorption spectroscopy at 405 nm using a conventional or microvolume spectrophotometer using ε = 10 700 M^{-1} cm^{-1}.

3.2. Before application, dilute the stock solution to a concentration of 1 mg/μL using the bath solution. Keep the stock solution at room temperature in the dark.

3.3. Labeling of endosomes and synaptic vesicles

3.3.1. To ubiquitously label endocytosed membrane compartments such as endosomes, add ND6 stock solution (e.g., 1 mg/μL in DMSO) to the culture medium at the final concentration of 1 μg/μL, and incubate at 5% CO_2, 100% humidity, and 37 °C for 30 min. To reduce the extent of SV labeling, suppress the spontaneous neuronal activity pharmacologically. For example, use tetrodotoxin to block action potentials or 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[f]quinoxaline-7-sulfonamide (NBQX) and D-(-)-2-amino-5-phosphonopentanoic acid (D-AP5) to inhibit excitatory transmission[23].

3.3.2. To selectively label SVs, use brief but strong stimulation to evoke presynaptic exo-/endocytosis. First, transfer the culture coverslip(s) to a 35 mm ∅ Petri dish containing 2 mL of normal Tyrode's solution at room temperature. Second, dilute the ND6 stock solution in high-K^+ Tyrode's solution (90 mM KCl) at the final concentration of 1 μg/μL. Third, replace the normal

Tyrode's solution in the Petri dish with ND6-containing high-K^+ Tyrode's solution and incubate at room temperature for 2 min.

3.4. Transfer the ND6-labeled cell culture to the imaging chamber filled with prewarmed normal Tyrode's solution containing 10 μM NBQX and 10 μM D-AP5.

3.5. Adjust the perfusion speed to ~0.2 mL/s (i.e., 1 drop per second) and start the perfusion of prewarmed normal Tyrode's solution containing NBQX and D-AP5 to remove excessive ND6 in the culture.

3.6. Adjust the focus and locate the appropriate field of view containing healthy and well-spread neurons bearing connected neurites. Avoid areas containing unresolved dye colloids.

3.7. Try imaging ND6-loaded cells with different exposure times to identify the best imaging settings.

NOTE: For the best exposure time, the highest pixel fluorescence intensity in the resulting image is about half of the bit range (e.g., for a 16-bit image, the bit range is from 0 to 65535), which will allow a further increase in fluorescence when the acidic bath solution is applied. The selected exposure time should be used for all ND6 imaging.

3.8. Set up the stimulation and perfusion protocol, frame interval, and total duration. For example, in the following protocol, use a 30 s baseline, a 10 s 30 Hz electric field stimulation, 50 s recovery, 120 s 90 mM K^+, 60 s recovery, 60 s Tyrode's solution with 50-mM NH_4Cl, 60 s Tyrode's solution at pH 5.5, and a frame interval of 3 s.

3.9. Start the image acquisition accompanied by the synchronized stimulation and perfusion. Monitor the simulation and solution exchange during imaging.

3.10. Stop the perfusion after the imaging ends, remove the coverslip, and clean the imaging chamber for the next trial.

4. Quantification of membrane trafficking by a change in ND6 fluorescence

4.1. Back up and/or make an electronic copy of all image files.

4.2. Choose an analysis program for data extraction, e.g., an open-source image analysis software such as ImageJ[24] and/or FIJI[25].

4.3. Open or import an image stack to the analysis program.

4.4. Set the first image as the reference and align the rest to it using functions/plugins such as **Rigid Registration**[26], which will mitigate artifacts due to xy-drifting. Refer to **Figure 8** for example images.

4.5. Average all images acquired during the 30 s pre-baseline to produce a reference image. Save a copy of this image for future reference.

4.6. Set the intensity threshold to generate a binary image from the baseline-averaged image.

4.7. Use Watershed in ImageJ or similar functions in another program to separate connecting neurites or cells.

4.8. Use the **Analyze Particle** function with appropriate area size and circularity to solicit regions of interest (ROIs) corresponding to cell membranes, endosomes, lysosomes, or synaptic boutons. Save all selected ROIs.

4.9. Select four background ROIs in cell-free regions within the field of view.

4.10. Measure the average pixel intensities for each ROI in every frame of the image stack, and export the results for statistical analysis.

4.11. Calculate the mean intensity of the background ROIs to obtain the baseline noise, which will be subtracted from all selected ROIs.

4.12. Average the three highest average intensities for every selected ROI during the application of pH 5.5 Tyrode's solution to obtain the maximal fluorescence intensity, which is defined as 100% for normalization.

4.13. Average the three lowest average intensities for every selected ROI during the application of 50 mM NH_4Cl to set the minimal fluorescence intensity, which is defined as 0% for normalization.

4.14. Calculate the relative fluorescence changes for every ROI based on its own 0% and 100% intensities. Derive mean fluorescence changes, change kinetics, and other values/plots from individual ROI data.

REPRESENTATIVE RESULTS:

SVs are specialized for neurotransmitter release via evoked exo-/endocytosis[27]. SVs have highly acidic lumen (i.e., pH 5.5), which is ideal for ND6. We used high K^+ stimulation to evoke SV exo-/endocytosis in order to allow ND6 to access SV. Expectedly, bright green fluorescent puncta along neuronal processes were visible after loading (**Figure 9A**). The line profile shown in **Figure 4B** demonstrated a substantial overlap between ND6 (green curve) and the FM4-64 puncta (red curve). The strong correlation between ND6 and FM4-64 fluorescence intensities also suggests SV staining by ND6 (**Figure 9B)**.

Electric stimulation and a high K^+ stimulation were used to evoke the release of the readily releasable pool (RRP, SVs with high release probability) and the reserve pool SVs (SVs with low

release probability), respectively. There were decreases in ND6 fluorescence in response to both stimuli (**Figure 4C** and **Figure 4E**), which suggests that ND6 resides in the SV membrane and that the SV lumen is neutralized (reported by ND6 signal decrease) during SV release.

At the end of every trial, 50 mM NH_4Cl was applied to deacidify SVs[28] and the pH 5.5 solution to brighten surface membrane **ND6** (**Figure 4D** and **Figure 4F**). The differences in fluorescence allow the detection of ND6 in the surface membrane (~44%) and SV membrane (~56%). These numbers match the fractions of surface and SV membranes at the axon terminals[29], suggesting that ND6 evenly distributes across membranes. Moreover, ND6 signals during two different stimuli and two pH manipulations allow the estimation that the short electric burst mobilized about ~31% SVs and high K^+ stimulation released ~70% of the remaining SVs. The rates of ND fluorescence decrease during the stimulations also match the time constant for the evoked SV exocytosis previously reported[30].

ND6's compact size leads to much less steric disturbance to cell membranes than a previously described tagging method[14] and thus offers a more accurate measurement of SV trafficking. Supporting that hypothesis, a 10x higher loading concentration showed no significant difference in FM4-64 destaining during stimulation (**Figure 10**). However, 1 μM is still recommended, given its moderate staining in astrocytes.

As SV exo-/endocytosis involves cholesterol (an abundant and vital lipid in the neuronal membranes), ND6 imaging was used to determine how membrane cholesterol affects SV release and retrieval. Treatment with 1 mM methyl-β-cyclodextrin (MβCD) for 90 min can remove ~10% cholesterol from neuronal surface membranes[31], mimicking aging-associated membrane cholesterol decrease. Under an exhaustive electric stimulation, the MβCD treatment significantly reduced SV release and retrieval measured by ND imaging (**Figure 11A**), which suggests that membrane cholesterol facilitates SV exo-/endocytosis[32]. We also evaluated cholesterol's contribution to SV pool replenishment, which is crucial for the fidelity of neurotransmission[33]. Two electric stimulations with a 10-s interval were applied in the presence of Bafilomycin A1 (BafA1). BafA1 selectively inhibits v-ATPase that reacidifies SVs. By acutely blocking the reacidification of retrieved SVs, BafA1 prevents ND6 fluorescence recovery after stimulation (**Figure 11B**). The ND6 decrease during the second stimulus should only be due to nascent SVs that replenish the empty RRP. Compared to the sham control, a significantly smaller ND6 response to the second stimulus was observed in the neurons pretreated with MβCD (i.e., smaller amplitude and faster decay of ND6 fluorescence reduction). This result supports the notion that cholesterol plays a pivotal role in recruiting new SVs to RRP.

FIGURE AND TABLE LEGENDS:

Figure 1: General synthesis scheme for ND6 probe. This figure has been modified from Thomas et al.[20].

Figure 2: Properties of ND6. (**A** and **B**) Solvatochromic properties of ND6. Absorbance spectra (**A**) and fluorescence spectra (**B**) in various solvents excited at 405 nm. (**C**) Comparison of

fluorescence intensity of ND6 in water (red) and 1% octyl glucoside solution at 1 µM. (**D**) ND6 fluorescence as a function of pH in 1% OG solution is proportional to protonation state of piperazine head group (calculated pKa = 7.4). Inset shows calculated protonation state of ND6 piperazine moiety (predicted pKa = 8.83). The dashed line represents fitted values. This figure has been modified from Thomas et al.[20]. Abbreviations: DCM = dichloromethane; MeCN = acetonitrile; DMSO = dimethylsulfoxide; EtOH = ethyl alcohol; MeOH = methyl alcohol; Abs= absorbance; Em = emission; OG = octyl glucoside.

Figure 3: Snapshot from molecular dynamics simulation trajectory. Interaction of ND6 probe in POPC membrane (left panel). Piperazine head group interacts strongly with phosphate groups (right panel) through electrostatic interactions. The black arrow (right panel) points at the C-N bond between the naphthalimide ring and piperazine. Results show that the piperazine group moves only slightly with a preference for the dihedral angle (atoms showed) between 90 and 120 degrees while maintaining its chair conformation. This figure has been modified from Thomas et al.[20].

Figure 4: ND6 labels synaptic vesicles and reports their release and retrieval in the nerve terminals. (**A**) Sample images of FM4-64 (red), ND6 (green), and overlay (yellow). The neurites and soma of one neuron were line-profiled. The straightened line images (20-pixel width) are next to the corresponding images. Arrowheads point to synaptic boutons marked by FM4-64. Scale bars = 100 µm. (**B**) Line profiles of FM4-64 and ND6 fluorescence intensities exhibit significant resemblance. (**C**) Sample traces of ND6 fluorescence changes at synaptic boutons indicated by arrowheads in **A** in response to stimuli. (**D**) Sample traces of ND6 fluorescence changes in response to NH_4Cl and pH 5.5 Tyrode's solutions. (**E**) The de-staining of FM4-64 is temporarily coupled to ND6 intensity changes. Data are plotted as mean ± S.E.M. (**F**) ND6 fluorescence intensity but not FM4-64 intensity was decreased and increased by the applications of 50 mM NH4Cl and pH 5.5 Tyrode's solutions, respectively. Data are plotted as mean ± S.E.M. This figure has been modified from Thomas et al.[20].

Figure 5: Imaging setup based on a Nikon-TiE inverted microscope. Annotated are four components required for live-cell fluorescence imaging.

Figure 6: Stimulation-imaging setup. Setup modified from a Warner Instruments RC-26 chamber and PH-1 heating platform for temperature control and solution exchange.

Figure 7: Diagram of device configurations for image acquisition with synchronized stimulations and solution exchanges.

Figure 8: Sample images demonstrating the key steps in image analysis.

Figure 9: ND6 highlights synaptic vesicles clustered at presynaptic terminals. (**A**) Sample images FM4-64 (red) and ND6 (green) co-loading at high magnification. Scale bar = 30 µm. (**B**) Scatter plot of FM4-64 and ND6 mean fluorescence intensities at the same ROIs corresponding to synaptic boutons and linear regression fit. r = 0.8353; $p = 1.7 \times 10^{-8}$; fields of view N = 9; ROIs n =

450. The threshold for FM4-64 is 1200 au (arbitrary unit) and 160 au for ND6. This figure has been modified from Thomas et al.[20]. Abbreviation: ROIs = regions of interest.

Figure 10: ND6 does not intervene with SVs. (**A**) ND6-represented SV turnover (by electric stimulus) after 1 or 10 µM ND6 loading. (**B**) FM4-64-measured SV turnover (with the electric stimulus). This figure has been modified from Thomas et al.[20]. Abbreviation: SVs = synaptic vesicles.

Figure 11: Cholesterol reduction impairs SV turnover. (**A**) ND6-represented SV turnover under 10 Hz, 120 s electric stimulus in control and MβCD-treated neurons. (**B**) Bafilomycin A1 prevents the reacidification of recycled SVs (i.e., no ND6 fluorescence recovery after stimulation-evoked decrease) and further elucidates MβCD's impact on SV replenishment. This figure has been modified from Thomas et al.[20]. Abbreviation: SV = synaptic vesicle; MβCD = methyl-β-cyclodextrin; BafA1 = Bafilomycin A1.

Figure 12: C2 was injected into CA3 of a C57BJ mouse. An acute coronal hippocampal slice was imaged by a 2P microscope using 760 nm excitation. Fluorescent puncta in the zoom-in image on the left show that C2 was intracellularly transported to the luminally acidic membrane compartment in distal neurites. Scale bars = 100 µm.

DISCUSSION:

Lipid-based dyes, such as 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI) and 3,3'-Dioctadecyloxacarbocyanine perchlorate (DiO), have long been used to illustrate cell morphology and track cellular processes such as the axon projections of neurons. Styryl dyes, such as FM1-43, have been invented and used successfully for the study of exocytosis[34]. Due to their low membrane affinity, they selectively label endocytosed vesicles where they are trapped while dyes remaining on the plasma membrane are washed off by constant perfusion. As such, styryl dyes are not suitable for continuous monitoring of vesicle recycling.

The recent invention of pH-sensitive GFP (i.e., pHluorin) made it possible to repeatedly visualize vesicle recycling when tagged to a vesicular membrane protein such as VAMPII or Synaptophysin[35]. Following the same principle, tagging a pH-sensitive fluorophore to lipid molecules enables the tracking of lipid membrane recycling during cycles of exo-/endocytosis[14]. In this case, being a single entity of lipid, ND6 is easier to prepare, simpler to apply, more efficient to label lipid membranes, less disruptive to cells, more stable in staying in cell membranes, and thus more reliable for fluorescence imaging. Its membrane-dependency and inverse pH-sensitivity allow brighter staining of acidic organelles such as SVs and endosomes.

Moreover, it is also feasible to label such acidic organelles or presynaptic terminals in tissues such as hippocampal slices as unspecifically distributed ND6 is quenched by neutral pH in extracellular or cytosolic spaces (**Figure 12**). Additionally, its large Stokes shift makes ND6 ideal for multiphoton imaging of deep tissues. Therefore, ND6 and other pH-sensitive lipid-based fluorophores allow the real-time optical measurement of lipid and cell membrane trafficking between the plasma membrane and intracellular apparatus such as SVs and endosomes for

multiple rounds of exo-/endocytosis. Given that synapses and SVs are vital to neurotransmission, ND6 is undoubtedly useful for studying synaptic physiology.

Given the modular design of these lipid analogs, it is feasible to conjugate ND to other lipids, such as phospholipids and sphingolipids, in various types of cell membranes or organelles. Moreover, the ND groups can be replaced with other environment-sensitive fluorophores to detect other environmental factors such as calcium or zinc concentration inside or outside cells or organelles. Furthermore, fluorophores with different emission spectra can be linked to membrane lipids to expand the palette of lipid reporters. For all those modifications, the linker between lipids and ND or other fluorescent groups can be adjusted to achieve better photo-properties and/or desired sensitivities.

This protocol describes the use of ND6 in visualizing SV turnover using live-cell imaging. Critical steps include loading, synchronized stimulations, and fluorescence quantification, all of which significantly affect the quality of the results. Moreover, the parameters/settings used in those steps can be modified according to the needs of the study. For example, the stimulation during the loading can be adjusted (shorter or longer duration) to allow the access of different pools of SVs (pools with high or low releasable probabilities, respectively). For live-cell fluorescence imaging, it is important to strike a balance between cell health and fluorescence intensity, which is particularly important here. This is because the excitation for ND6 is near-UV (i.e., 405 nm), which can cause more phototoxicity and dye breakdown than visible light. Thus, it is important to adjust the excitation power, exposure time, frame rate, and imaging duration to minimize photodamage and maximize the signal quality.

ND6 is a very interesting probe. Its large Stokes shift made it possible to use it simultaneously with other membrane dyes such as FM4-64[20]. More importantly, it is a suitable donor candidate for fluorescence resonance energy transfer (FRET) and can be excited by purple light, which will be much less likely to co-excite the FRET recipient. Lipid-mimicking dyes, such as ND6, make it possible to study the interactions between membrane proteins and lipids during exo-/endocytosis. The pH-dependent association and dissociation between membrane proteins and lipids will be magnified in the acidic lumen of SVs or endosomes, facilitating the exploration of the role of membrane lipids in receptor-mediated endocytosis and sorting. In summary, ND6 and its derivatives can significantly expand the toolbox in studying membrane lipids and their trafficking in live cells.

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DISCLOSURES:
The authors declare no competing financial interests.

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Figure1

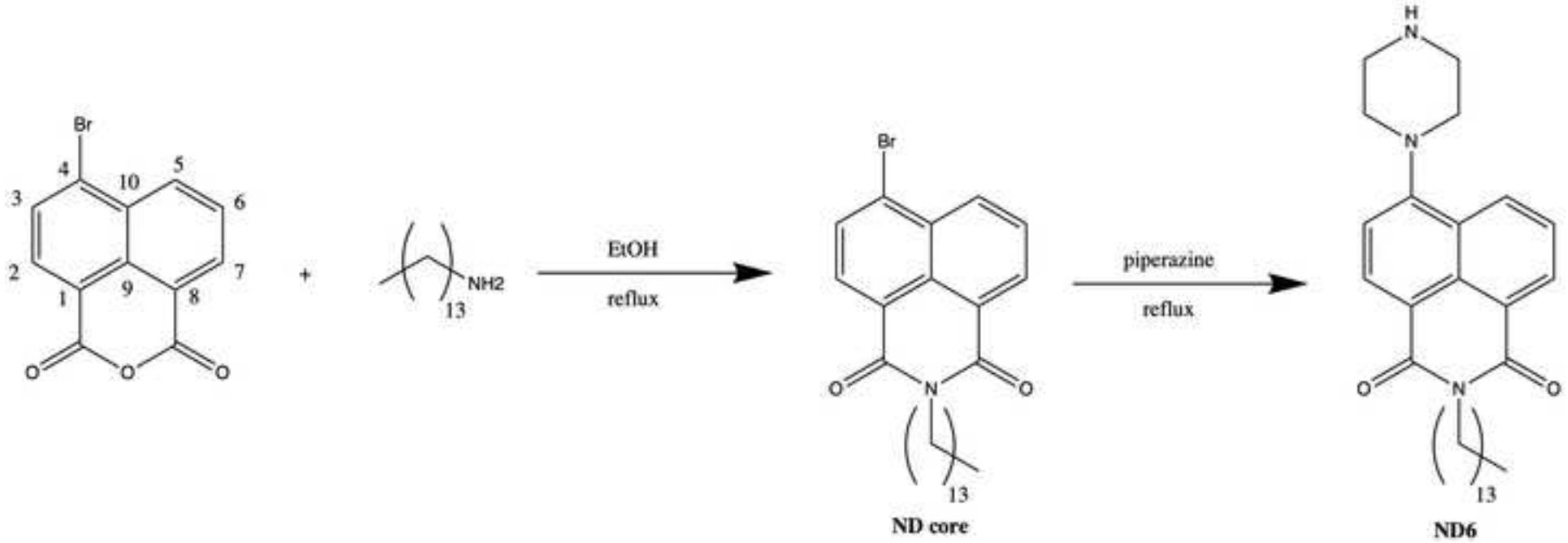

Figure 2

Click here to access/download;Figure;Figure02.jpg

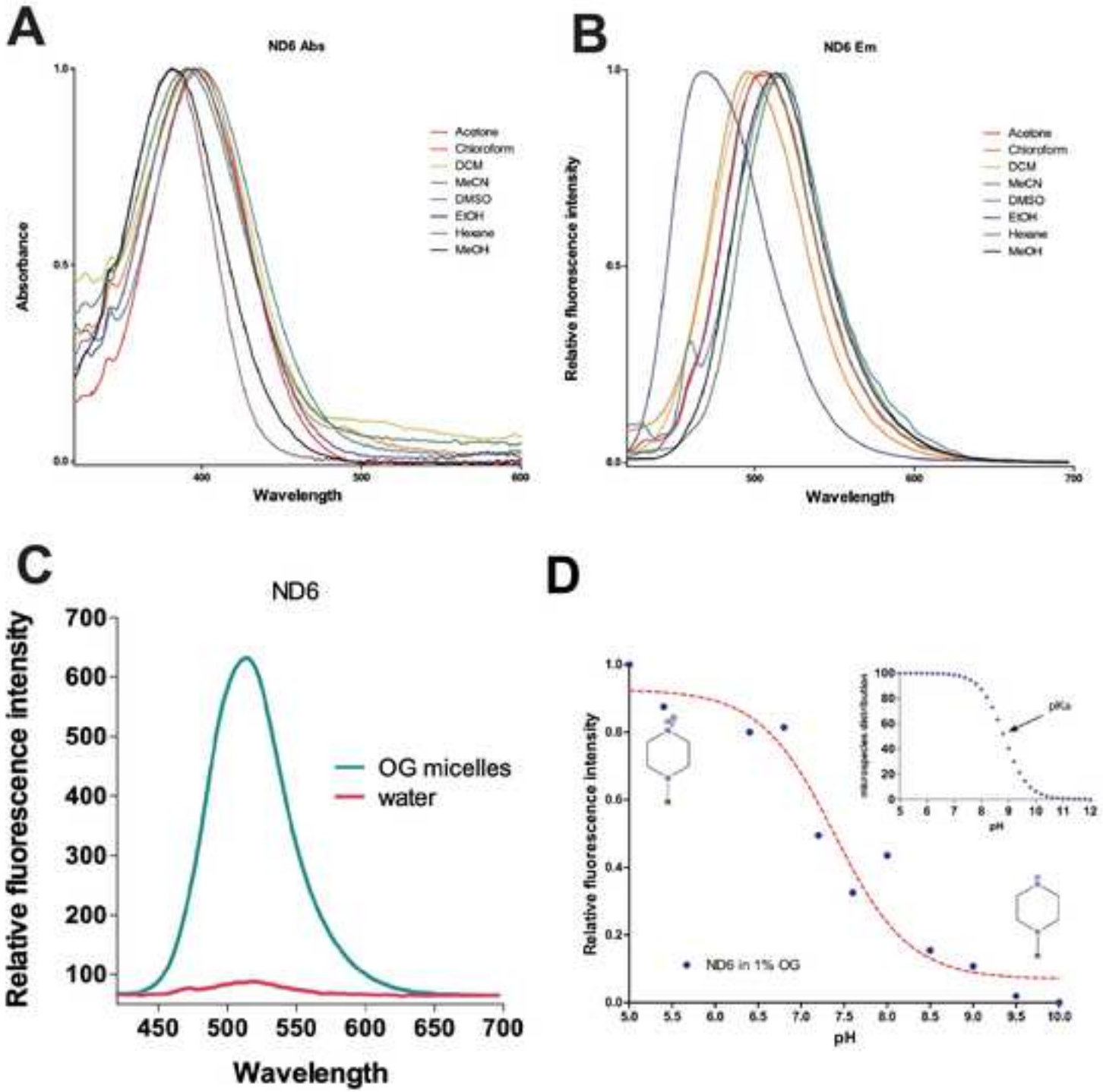

Figure3

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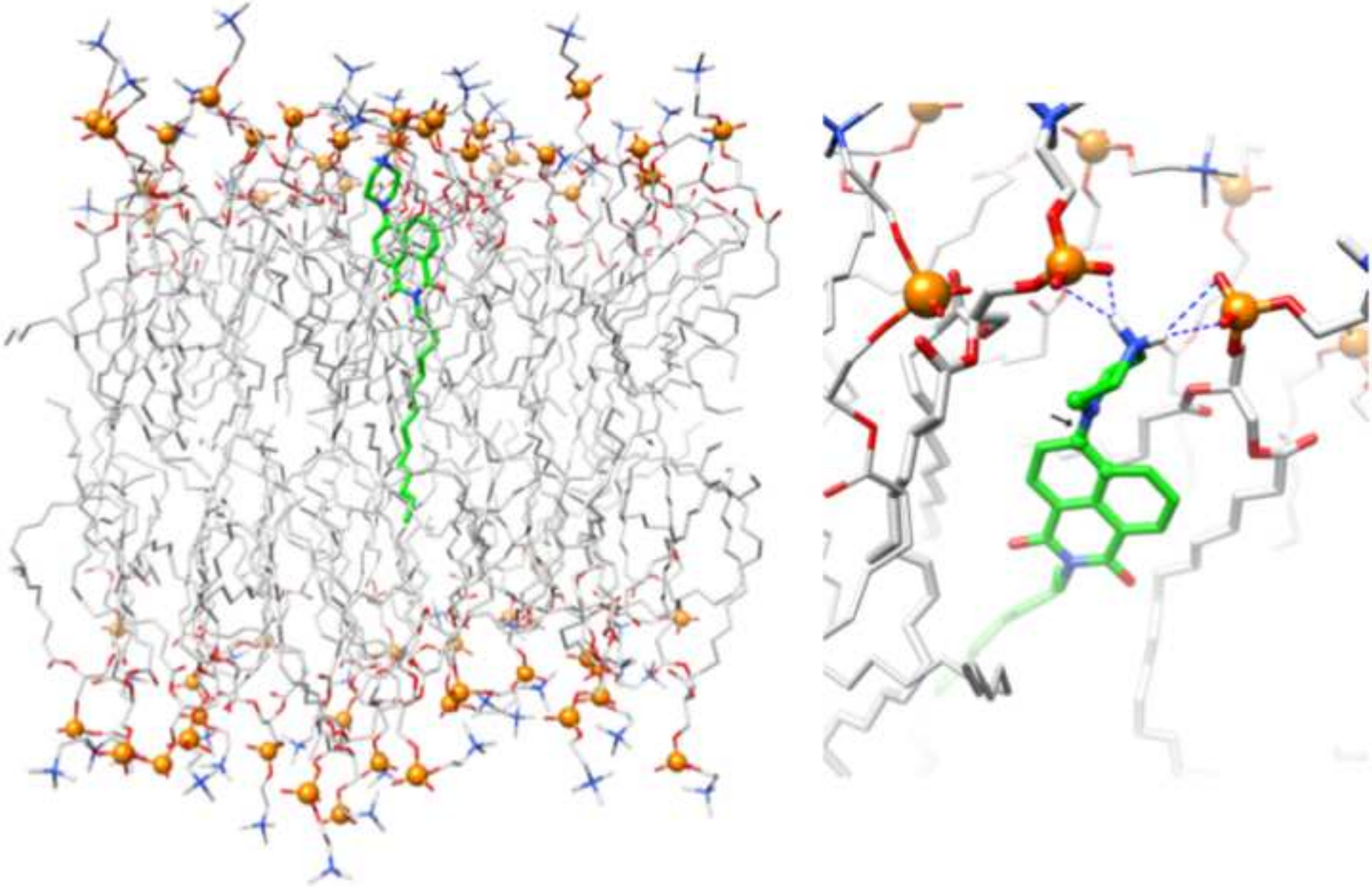

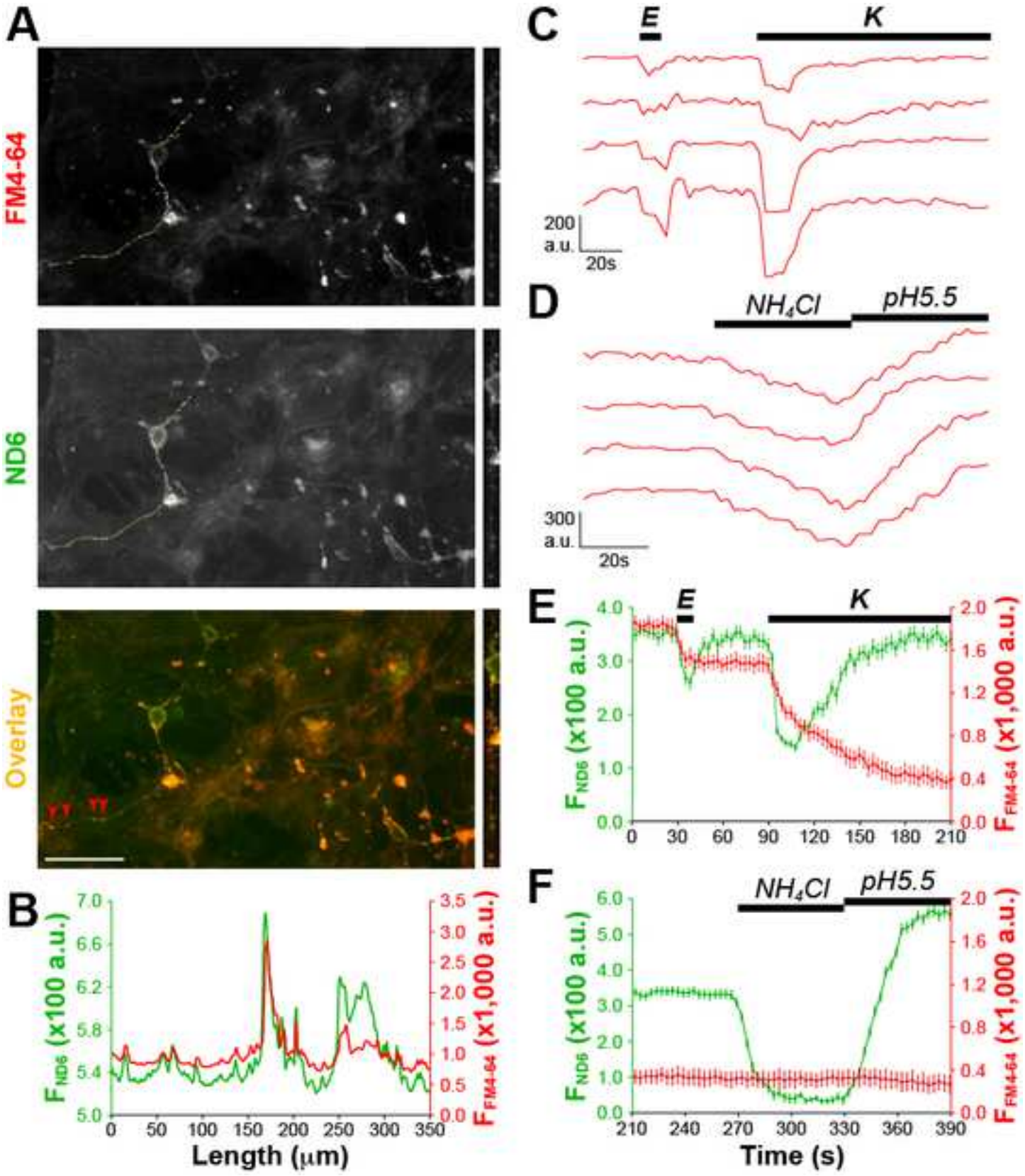

Figure5

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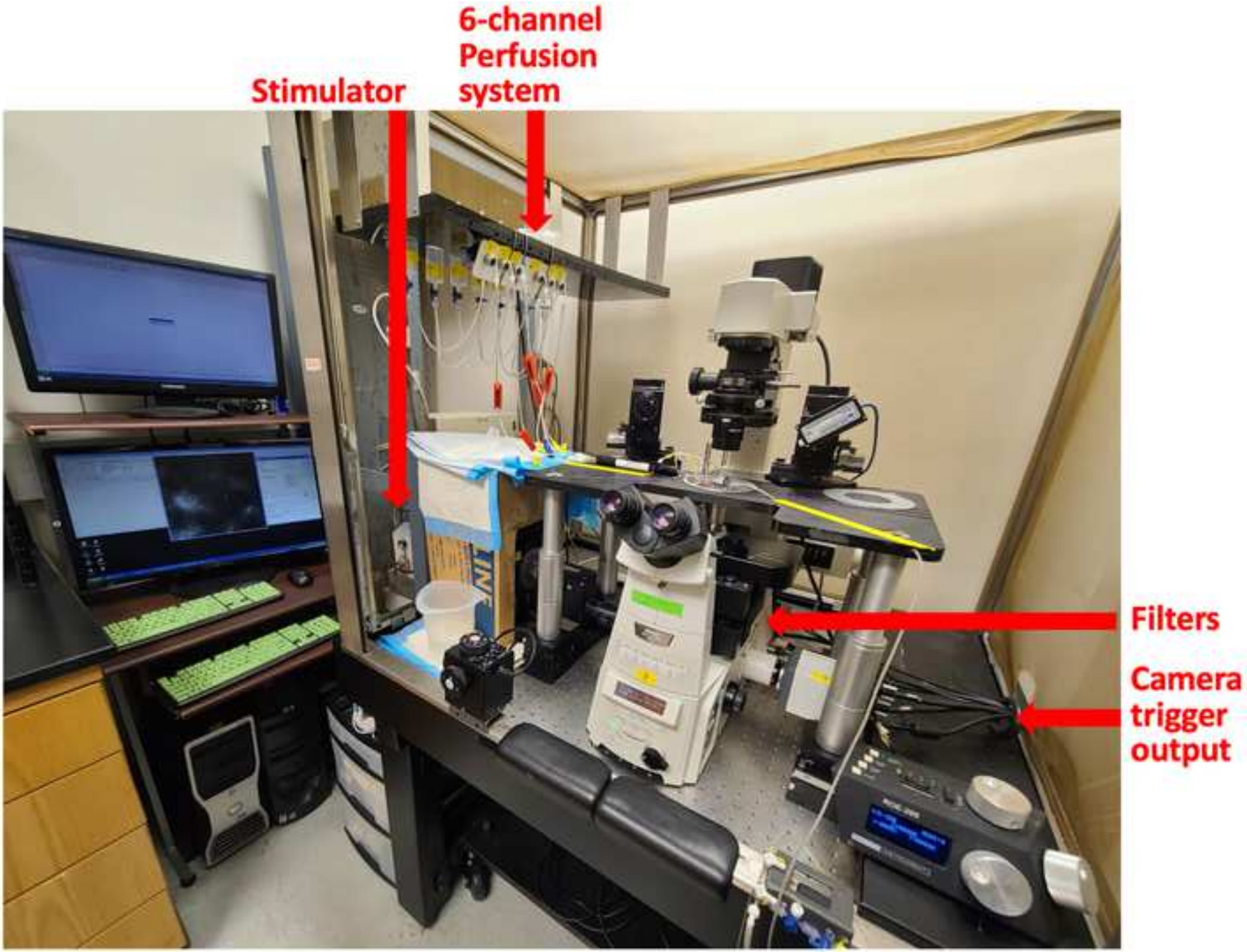

Figure6

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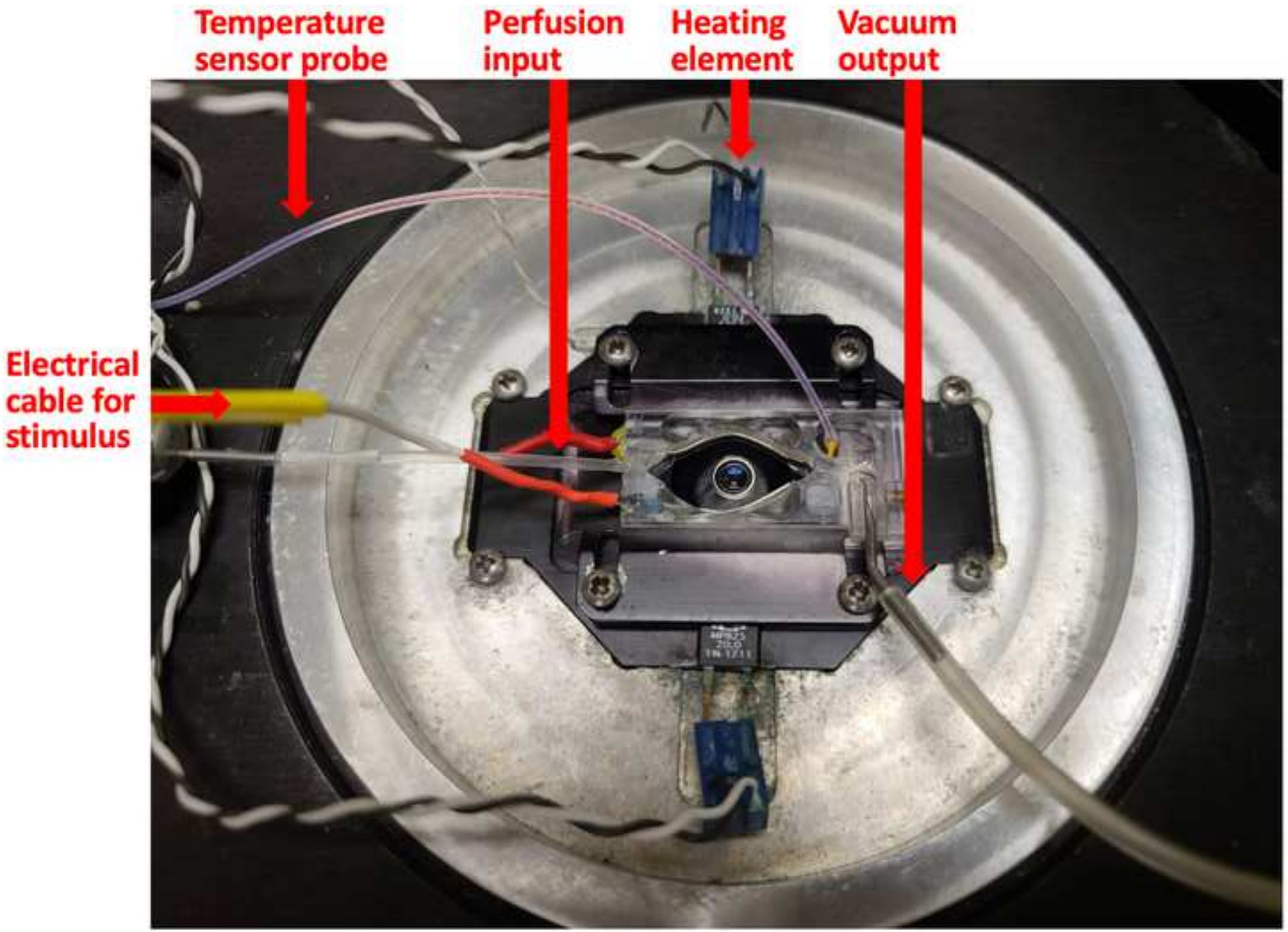

Figure7

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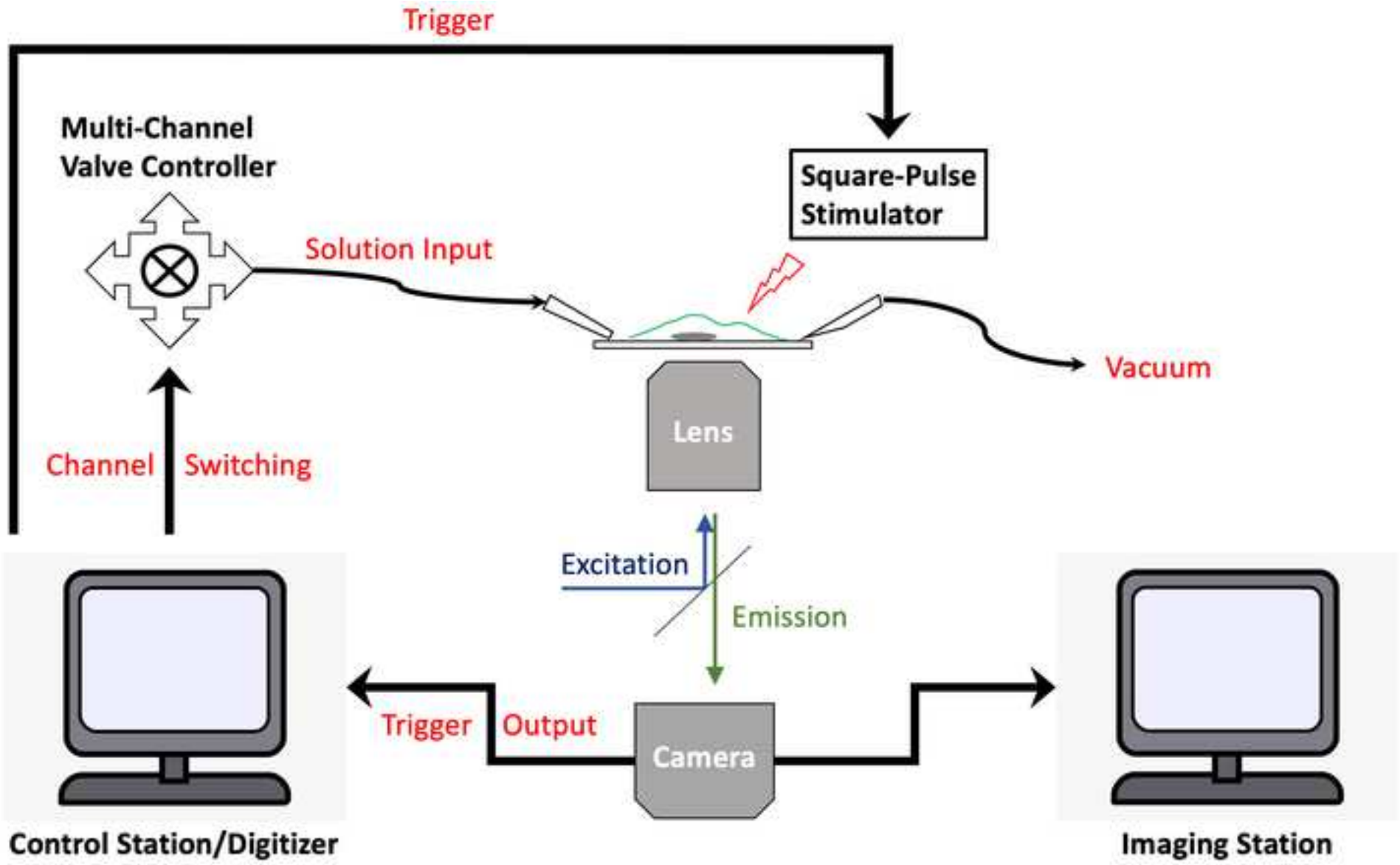

Figure8

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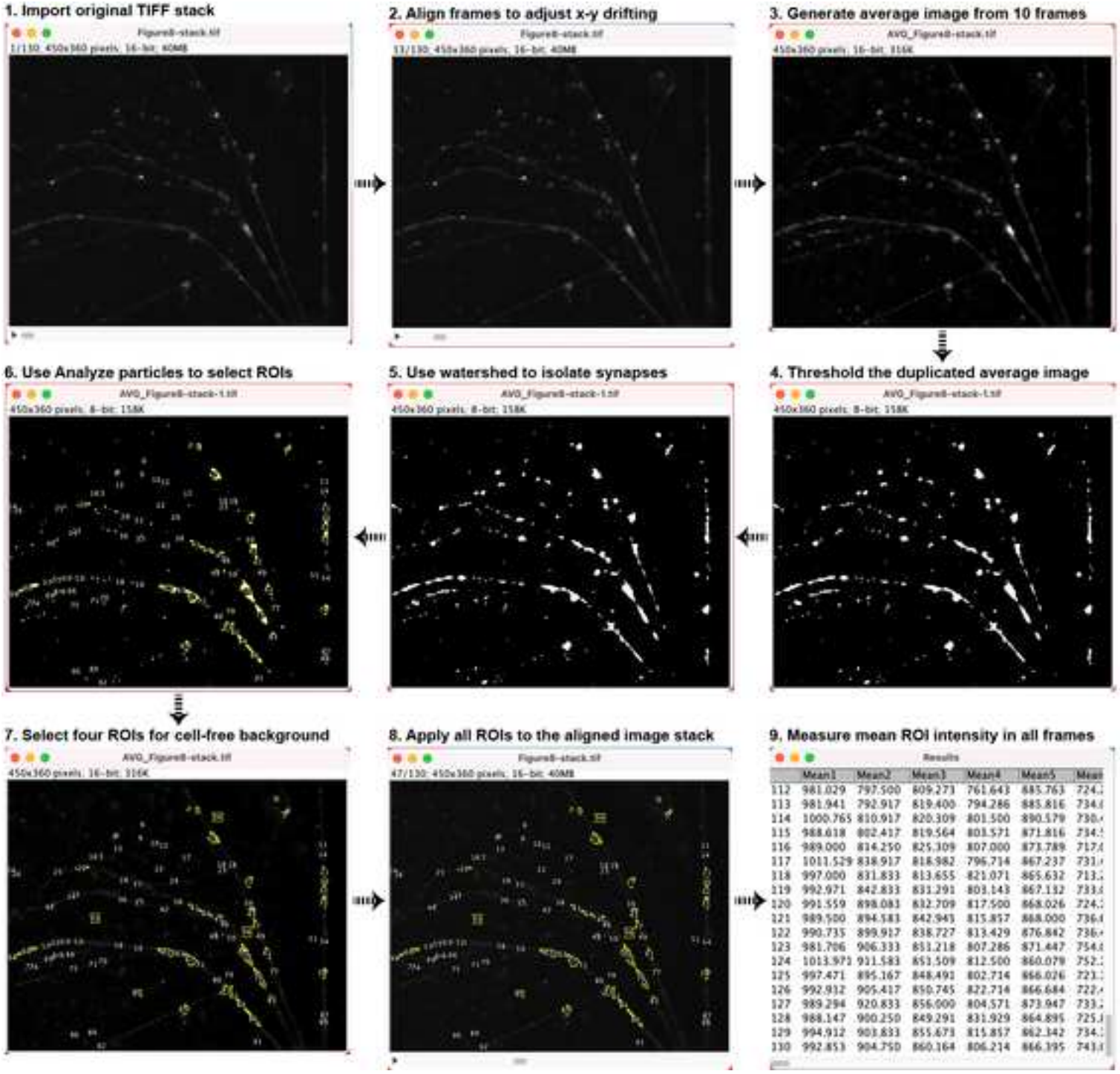

Figure9

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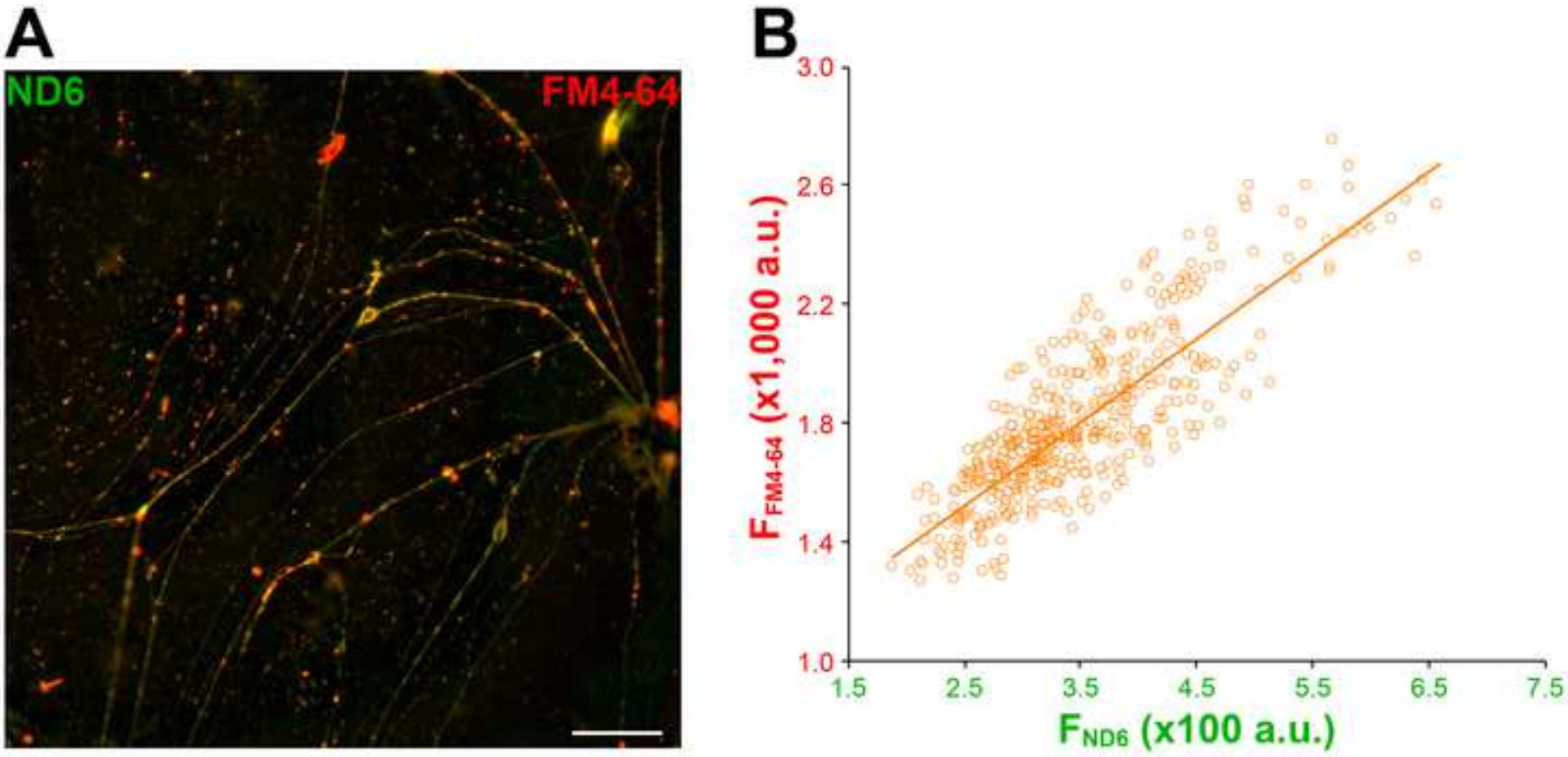

Figure10

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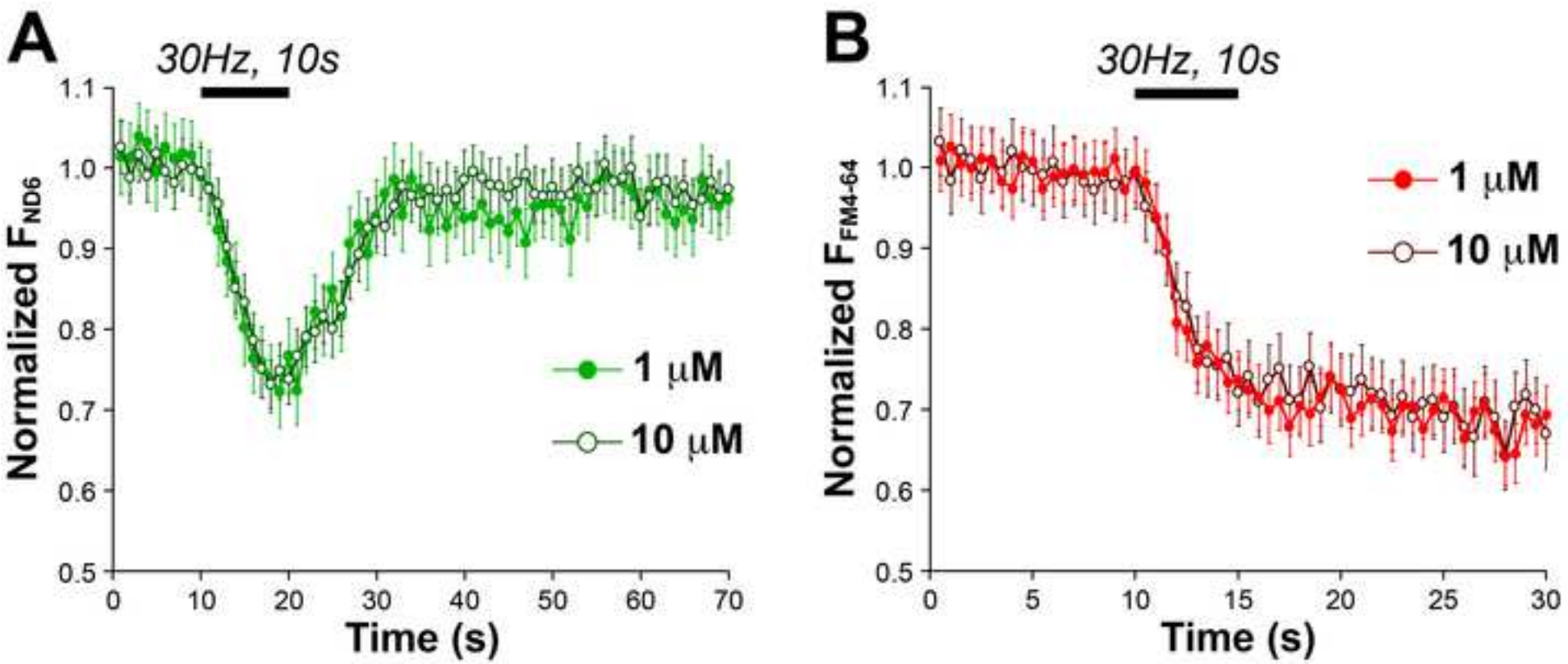

Figure11

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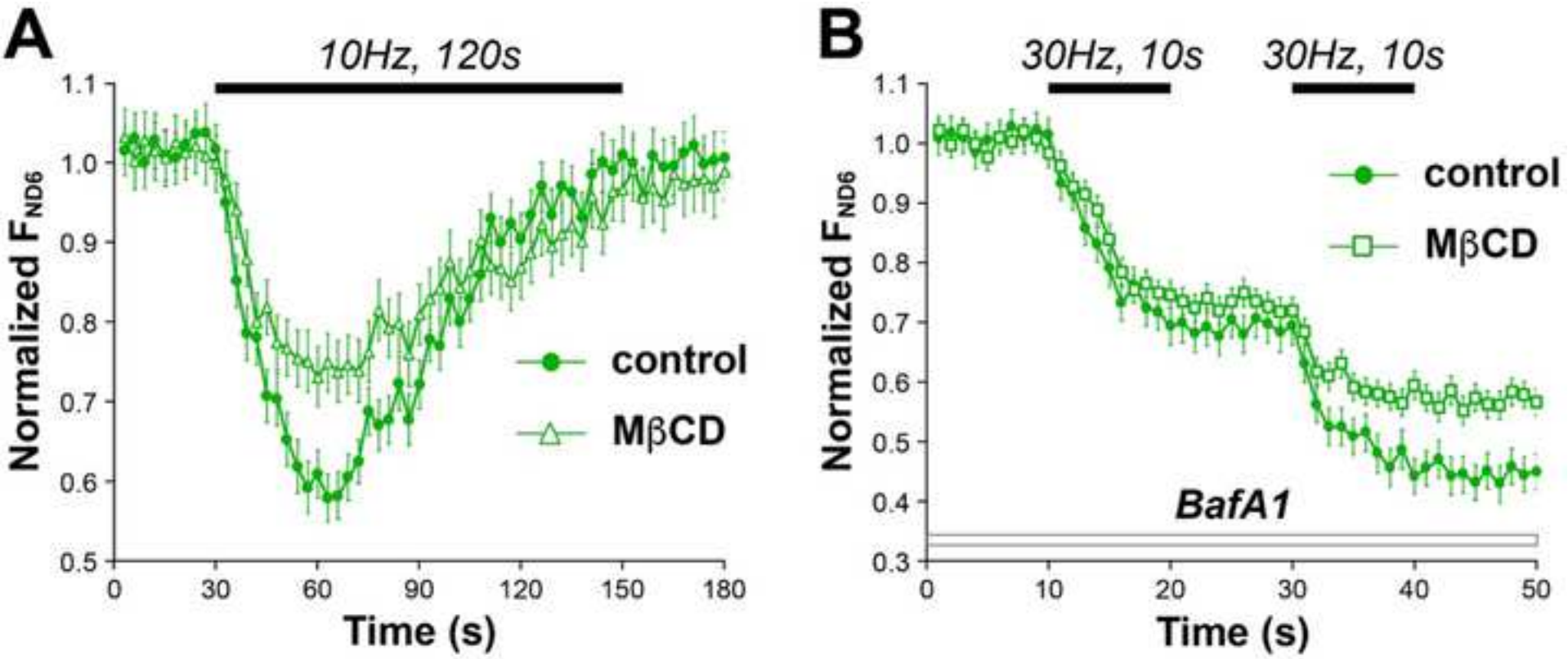

Figure12

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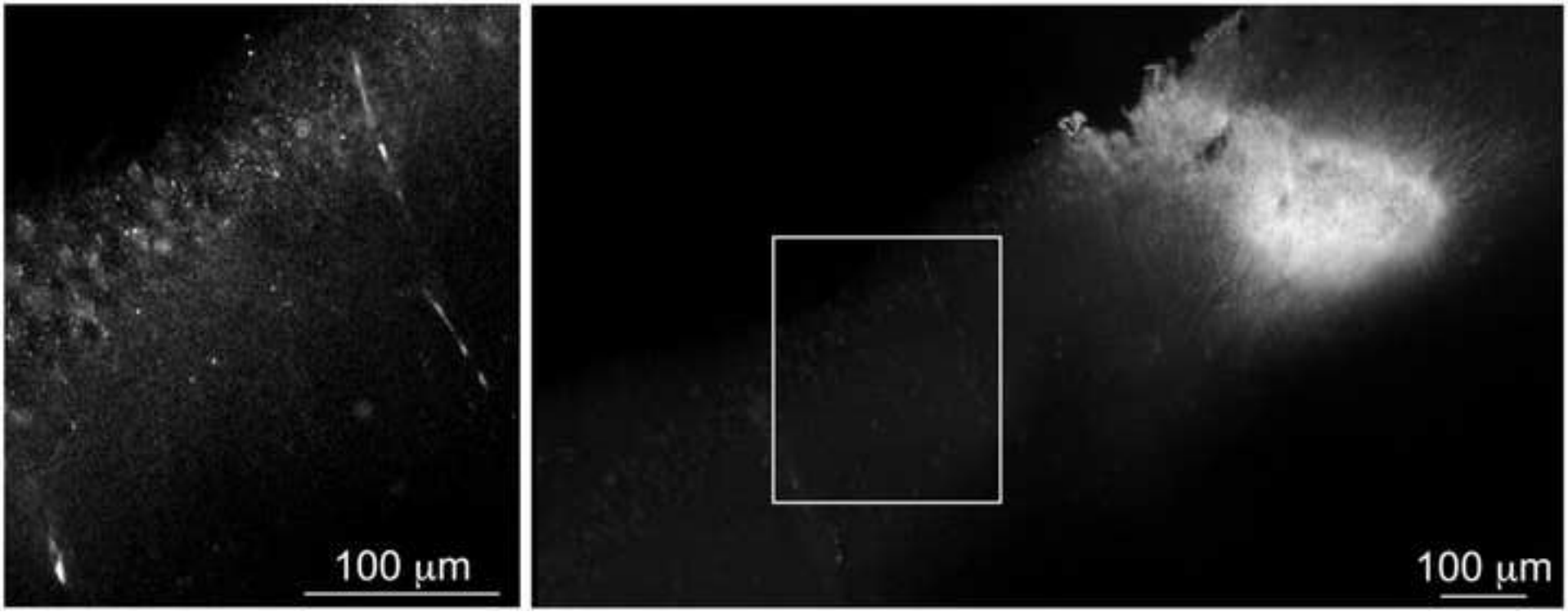

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Table of Materials

Table of Materials-QZ06112021.xlsx

Dear Dr. Vineeta Bajaj:

Thank you for considering our manuscript. And we are very grateful for all reviewers' comments. Below you will find our point-by-point responses to these comments/concerns. In the revised manuscript, all changes in the text have been marked in red fonts.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have done our best to correct errors in spelling and grammar.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have modified the format accordingly.

3. Please provide an email address for each author.

Email addresses have been added to the author list.

4. Please include a Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "This protocol presents…"

The description has been added to the second page.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."

We have revised the protocol section accordingly.

6. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

We have added more details to the protocol steps accordingly.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

We have re-numbered the protocol section accordingly.

8. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

We have changed the protocol text accordingly.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
We have simplified the steps accordingly.

10. Please ensure that you specify the volumes and concentration of the solutions used.
We have provided the specifications accordingly.

11. 1.4: How do you perform the dissection?
We have added a brief description of the dissection steps in the revision.

12. 2: For this section please include knob turns, button clicks etc to show how the steps are performed.
We have included them in the protocol section accordingly.

13. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Matrigel, Nikon, Hamamatsu Flash4.0 sCOM, Micro-manager, Warner Instrument RC-26, Digidata, PH-1 heating platform, Flash4.0, NanoDrop, Clampex, Axon DigiData 1440A, etc.
We have removed commercial languages and referenced them only in the Table of Materials and Reagents accordingly.

14. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Essential steps for the video are highlighted in green.

15. Please revise the representative result section to avoid overlap with previously published work.
We have revised that section accordingly.

16. All figures should be uploaded separately (all panels combined into one image file) to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text. Please do not include figure legend with the figure.
We have removed all figures from the manuscript and will upload them with designated names.

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The related paper was published in ACS Chemical Neuroscience. It provides copyright permission *via* its online request form instead of a letter from the editor. Its copyright policy can be found here (https://pubs.acs.org/page/copyright/permissions.html). We have obtained a formal permission *via* its website and attached it with our resubmission. For cited figures, we have referred the related paper accordingly in the figure legends, according to permission requirements.

18. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
While the last two were covered in the original manuscript, we have added the discussions for a), b), and c) in the revised discussion section.

19. Please do not abbreviate the journal titles in the reference section.
We have revised the reference list using JoVE format.

20. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file.
We have revised the table.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript, Alamgir, S. et al. described a detailed protocol of using a class of new pH-sensitive fluorescent lipid analogs for tracing lipid recycling between the plasma membrane and synaptic vesicles in cultured neurons.

This study is an important extension of the authors' previous work (Thomas, D. et al., 2021, ACS Chemical Neuroscience), in which they reported the synthesis, characterization, and application of a series of membrane- and pH-sensitive probes suitable for live-cell fluorescence imaging.

Major Concerns:

The experimental design and description are clear, and the experiments are well executed. Finally, the figures are clearly plotted and easy to understand. I don't have major criticisms but have some minor queries and comments, and hope the authors could address.

We highly appreciate the reviewer's fair assessment of our manuscript.

Minor Concerns:

1. Will the ND6 dye affect the cell physiology? The author should discuss about this and it would help readers.

Although we have not thoroughly test cell physiology, we have done cell viability assay (Tryptophan blue staining) and observed no significant difference between ND6 loaded group and sham control. Furthermore, we looked at ND6's impact on exocytosis at presynaptic terminals, which is of great relevance to its major application. Namely, we compared FM1-43 destaining at synaptic boutons of neurons treated with 1 and 10 uM of ND6. And the result (now Figure 10) showed no significant difference, suggesting that ND6 does not alter exocytosis. These tests and results were reported in our previous paper (Thomas, D. et al., 2021, ACS Chemical Neuroscience). Following the reviewer's suggestion, we have added this in the discussion.

2. How about the labeling efficiency of ND6? It seems the labeling efficiency is very low as shown in Figure 4A?

Figure 4A are sample images showing co-staining of ND6 and FM4-64. FM4-64 is a red fluorescent dye selectively and effectively labeling active axon terminals (i.e., presynaptic terminals actively release neurotransmitters *via* evoked exo- and endocytosis) (Betz, W.J., et al., 1996, Current Opinion Neurobiology). Due to its unique pH-sensitivity, ND6 fluorescence puncta represent presynaptic terminals packed with luminally acidic synaptic vesicles as well as large endosomes in neurons and astrocytes. As demonstrated by line profiling (Figure 4B), both fluorescence signals are well overlayed. Notably, the signal-to-noise ratio of ND6 is apparently better than that of FM4-64. Hence, we can conclude that the labeling efficiency of ND6 is as good as that of FM4-64 if not better.

3. For primary culture preparation, were all of the experiments done at room temperature? For example, in line 116, I would expect the digestion procedure was done at 37℃?
Yes, the Trypsin/EDTA treatment was done at 37 °C in the incubator (Also, 5% CO2 in the incubator helps to stabilize the pH of HBSS).

4. Figure 4: what is the difference between Figure 4C&4D and Figure 8? How about only showing the A and B in Figure 4 to indicate that ND6 has the ability label synaptic vesicles (similar as FM4-64), or remove the Figure 8?
We apologize for the mistake. The original Figure 8 has been removed.

5. Typos: Line 69 & line 295, "strokes"? Do you mean "stock"?
It should be “Stokes shift”. We are sorry about the mistake; it has been corrected.

Reviewer #2:
Manuscript Summary:
Zhang et al describe a protocol for measuring membrane lipid turnover. These measurements were described in reference 20 (ACS Chemical Neuroscience. 12 (4), 719-734, (2021)) and are based on a synthetized lipid analogue probe ND6 exhibiting pH-sensitivity and solvatochromism. The protocol is composed of four parts: cell culture, fluorescence imaging, cell labeling and quantification by image analysis.
Partitioning of ND6 between synaptic vesicles and plasma membrane was estimated in living neurones under stimulation to evoke the release of readily releasable poll (RRP) or the release of the reserve pool of synaptic vesicles. A decrease of exo/endocytosis was detected under membrane cholesterol scavenging. A lower recovery of RRP of synaptic vesicles was observed under simultaneous reacidification inhibition of the retrieved synaptic vesicles.

Major Concerns:
- The part (2) of the protocol "Set-up microscope for live-cell fluorescent imagiing" would benefit from additional details about synchronization of hardware and softawre (e.g. relying on a diagram).
We have added those details and a diagram in the revision.

- The part (4) of the protocol "Quantification of membrane trafficking by ND6 signal" should present a sample of images at different stages of processing.
Following the reviewer’s comment, in the revised version of the manuscript we have added a new figure containing those sample images.

- Line 84: fluorescence increase seems to be closer of two-fold than of three-fold in figure 2C

We apologize for the error and it has been corrected in the revised text. Between pH 5.5 (vesicle lumen) and pH 7.5 (extracellular environment), the difference is about 0.6 (normalized unit), which is two-fold fluorescence change.

-Representative results:
(i) Fluorescence ratios used to determine partitioning of ND6 are recalled from ref 20 p. 726. It should be useful to recall the time constant calculated from the fluorescence data in ref 20 as well. These time constants are relevant to quantify the dynamics of turnover.
Following the reviewer's comment, we have included the time constants from ref. 20 in the revised manuscript.

(ii) Figures 10A, 10B and 10C are missing. I guessed they are figures 12G and 12H in reference 20?
We apologize for the missing figures. They have been included in the revision.

- Discussion: figure 11 is missing
We apologize for the mistake. It has been included in the revision.

Minor Concerns:
A few typos among others:
line 53: to -> tool
line 54: have quickly -> have been quickly
line 69 strokes shifts -> Stokes shifts
line 152: weigh ->weight
line 245: estimated -> estimate
line 295 strokes shifts -> Stokes shifts
We are sorry about these typos and they have been corrected in the revised manuscript.

Reviewer #3:
Manuscript Summary:
This method describes the use of a lipophilic pH sensitive indicator that the authors first described in an article this year in ACS Chemical Neuroscience. In that article they created solvatochromatic dyes on an 1,8-naphthalimide fluorophore scaffold and one of them, ND6 whose use is discussed in this article, shows a substantial fluorescence difference between pH 6 and 7. This enables ND6 to be used as a pH sensor in the surrounding of lipid in which the sensor is embedded.

Major Concerns:
1) The important details of how to label different subcellular compartments appear a little lost in the details of the manuscript. This may be more a matter for how JOVE likes to present material, but for me too much of the methods relate to relatively generic imaging and cell culture preparation. As described the application of the dye should be

successful in any number of preparations and on a variety of microscope approaches. Indeed, the authors briefly discuss its use in slice tissue. Consequently, while the cell culture and imaging approaches described seem perfectly reasonable their detailed description takes away somewhat from the utility of using this class of dyes.
In this protocol, we are focusing on using ND6 to visualize the trafficking of membrane lipids during exo-/endocytosis cycle, a fundamental cellular process particularly vital to neurotransmission at chemical synapses. Following the reviewer's comments, we have expanded the discussion about the utility of this dye.

2) A possible advantage of ND6 over a genetically engineered probe, such as a pHluorin is the enhanced signal and photostability. If this is the case, then faster fusion readouts could be obtained. It is hard to see from the given examples, what temporal resolution could be achieved. This is not so much a criticism of the presented work, but in terms of understanding the importance and potential of the work, pushing the spatiotemporal limits might be helpful.
We have been using 3-s frame interval for image acquisition. This is because (1) the excitation light is near UV (i.e., 405 nm) which is damaging to cells; (2) the total imaging duration is about 390s for the imaging protocol mentioned in step 3.8. In an unpublished study, we have increased the frame rate to 5 Hz and decreased the duration to 20-s in order to resolve synaptic vesicle release during a 30-Hz 10-s electric stimulation.

3) From the data in figure 4/8 the dye appears to reacidify. The implication is that the dye can recycle in the membrane and be endocytosed. This is a particular advantage over FM dyes that may allow for rounds of restimulation. A discussion of this point would be nice
We agree with the reviewer and have added that in the discussion.

4) Fig 8 is a repeat of parts of fig 4. This is unnecessary
In this data to gain an idea of signal to noise it would be nice to see if changes at single synaptic bouton sized objects could be resolved
We apologize for the mistake and it has been corrected in the revision. The original Figure 4C&D are averaged trace of individual synaptic boutons identified by FM4-64 labeling. We have added sample traces from single synaptic boutons in Figure 4.

5) Both Figs 10 and 11 are referenced in the text but missing in the version I have. To assess these, I would need access.
We are sorry for the mistake. We resubmitted the two Figures and have added them in the revised manuscript.

Solvatochromic and pH-Sensitive Fluorescent Membrane Probes for Imaging of Live Cells

Author: Deborah Thomas, Vicente Rubio, Vijaya Iragavarapu, et al
Publication: ACS Chemical Neuroscience
Publisher: American Chemical Society
Date: Feb 1, 2021

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