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## Targeted Plasma Membrane Delivery of a Hydrophobic Cargo Encapsulated in a Liquid Crystal Nanoparticle Carrier

--Manuscript Draft--

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<b>Corresponding Author:</b>	James Delehanty US Naval Research Laboratory Washington, DC UNITED STATES
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author E-Mail:</b>	james.delehanty@nrl.navy.mil
<b>Corresponding Author's Institution:</b>	US Naval Research Laboratory
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	James Delehanty
<b>First Author Secondary Information:</b>	
<b>Other Authors:</b>	Okhil K Nag Jawad Naciri Eunkeu Oh Christopher Spillmann
<b>Order of Authors Secondary Information:</b>	
<b>Abstract:</b>	The controlled delivery of drug/imaging agents to cells is critical for the development of therapeutics and the study of cellular signaling processes. Recently, nanoparticles (NPs) have shown significant promise in the development of such delivery systems. Here, we have employed a liquid crystal NP (LCNP)-based delivery system for the controlled delivery of a water-insoluble dye, 3'-dioctadecyloxacarbocyanine perchlorate (DiO), from within the NP core to the hydrophobic region of plasma membrane bilayer. During synthesis of the NPs, the dye was efficiently incorporated into the hydrophobic LCNP core as confirmed by multiple spectroscopic analyses. Conjugation of a PEGylated cholesterol derivative to the NP surface (DiO-LCNP-PEG-Chol) enabled the binding of the dye-loaded NPs to the plasma membrane in HEK 293T/17 cells. Time-resolved laser scanning confocal microscopy and Förster resonance energy transfer (FRET) imaging confirmed the passive efflux of DiO from the LCNP core and its insertion into the plasma membrane bilayer. Finally, the delivery of DiO as a LCNP-PEG-Chol attenuated the cytotoxicity of DiO; the NP form of DiO exhibited ~30-40% less toxicity compared to DiO free delivered from bulk solution. This approach demonstrates the utility of the LCNP platform as an efficient modality for the membrane specific delivery and modulation of hydrophobic molecular cargos.
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**TITLE:**

**Targeted Plasma Membrane Delivery of a Hydrophobic Cargo Encapsulated in a Liquid Crystal Nanoparticle Carrier**

**AUTHORS:**

Okhil K. Nag, Jawad Naciri, Eunkeu Oh, Christopher M. Spillmann and James B. Delehanty

**AUTHORS: INSTITUTION(S)/AFFILIATION(S) FOR EACH AUTHOR:**

Okhil K. Nag  
Center for Bio/Molecular Science and Engineering, Naval Research Laboratory,  
Code 6900  
Washington, DC  
okhil.nag.ctr@nrl.navy.mil

Jawad Naciri  
Center for Bio/Molecular Science and Engineering, Naval Research Laboratory,  
Code 6900  
Washington, DC  
jawad.naciri@nrl.navy.mil

Eunkeu Oh  
Optical Sciences Division, Naval Research Laboratory,  
Code 5600  
Washington, DC  
Sotera Defense Solutions,  
Columbia, MD  
eunkeu.oh.ctr.ks@nrl.navy.mil

James B. Delehanty  
Center for Bio/Molecular Science and Engineering, Naval Research Laboratory,  
Code 6900  
Washington, DC  
james.delehanty@nrl.navy.mil

**CORRESPONDING AUTHOR:**

James B. Delehanty, Ph.D.

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**SHORT ABSTRACT:**

A liquid crystal nanoparticle (LCNP) nanocarrier is exploited as a vehicle for the controlled delivery of a hydrophobic cargo to the plasma membrane of living cells.

## LONG ABSTRACT:

The controlled delivery of drug/imaging agents to cells is critical for the development of therapeutics and for the study of cellular signaling processes. Recently, nanoparticles (NPs) have shown significant promise in the development of such delivery systems. Here, a liquid crystal NP (LCNP)-based delivery system has been employed for the controlled delivery of a water-insoluble dye, 3'-dioctadecyloxacarbocyanine perchlorate (DiO), from within the NP core to the hydrophobic region of a plasma membrane bilayer. During the synthesis of the NPs, the dye was efficiently incorporated into the hydrophobic LCNP core, as confirmed by multiple spectroscopic analyses. Conjugation of a PEGylated cholesterol derivative to the NP surface (DiO-LCNP-PEG-Chol) enabled the binding of the dye-loaded NPs to the plasma membrane in HEK 293T/17 cells. Time-resolved laser scanning confocal microscopy and Förster resonance energy transfer (FRET) imaging confirmed the passive efflux of DiO from the LCNP core and its insertion into the plasma membrane bilayer. Finally, the delivery of DiO as a LCNP-PEG-Chol attenuated the cytotoxicity of DiO; the NP form of DiO exhibited ~30-40% less toxicity compared to DiO<sub>free</sub> delivered from bulk solution. This approach demonstrates the utility of the LCNP platform as an efficient modality for the membrane-specific delivery and modulation of hydrophobic molecular cargos.

## INTRODUCTION:

Since the advent of interfacing nanomaterials (materials  $\leq 100$  nm in at least one dimension) with living cells, a continuing goal has been to take advantage of the unique size-dependent properties of nanoparticles (NPs) for various applications. These applications include cell and tissue labeling/imaging (both *in vitro* and *in vivo*), real-time sensing, and the controlled delivery of drugs and other cargos<sup>1</sup>. Examples of such relevant NP properties include the size-dependent emission of semiconductor nanocrystals (quantum dots, QDs); the photothermal properties of gold nanoparticles; the large loading capacity of the aqueous core of liposomes; and the ballistic conductivity of carbon allotropes, such as single-wall carbon nanotubes and graphene.

More recently, significant interest has arisen in the use of NPs for the controlled modulation of drugs and other cargos, such as contrast/imaging agents. Here, the rationale is to significantly enhance/optimize the overall solubility, delivered dose, circulation time, and eventual clearance of the drug cargo by delivering it as an NP formulation. This has come to be known as NP-mediated drug delivery (NMDD), and there are currently seven FDA-approved NP drug formulations for use in the clinic to treat various cancers and hundreds more in various stages of clinical trials. In essence, the goal is to “achieve more with less;” that is, to use the NP as a scaffold to deliver more drug with fewer dosing administrations by taking advantage of the large surface area:volume (e.g., hard particles, such as QDs and metal oxides) of NPs or their large interior volume for loading large cargo payloads (e.g., liposomes or micelles). The purpose here is to reduce the necessity for multiple systemically-delivered dosing regimens while at the same time promoting aqueous stability and enhanced circulation, particularly for challenging hydrophobic drug cargos that, while highly effective, are sparingly soluble in aqueous media.

Thus, the goal of the work described herein was to determine the viability of using a novel NP scaffold for the specific and controlled delivery of hydrophobic cargos to the lipophilic plasma membrane bilayer. The motivation for the work was the inherent limited solubility and difficulty in the delivery of hydrophobic molecules to cells from aqueous media. Typically, the delivery of such hydrophobic molecules requires the use of organic solvents (e.g., DMSO) or amphiphilic surfactants (e.g., Poloxamers), which can be toxic and compromise cell and tissue viability<sup>2</sup>, or micelle carriers, which can have limited internal loading capacities. The NP carrier chosen here was a novel liquid crystal NP (LCNP) formulation developed previously<sup>3</sup> and that had been shown previously to achieve a ~40-fold improvement in the efficacy of the anticancer drug doxorubicin in cultured cells<sup>4</sup>.

In the work described herein, the representative cargo selected was the potentiometric membrane dye, 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO). DiO is a water-insoluble dye that has been used for anterograde and retrograde tracing in living and fixed neurons, membrane potential measurements, and for general membrane labeling<sup>5-9</sup>. Due to its hydrophobic nature, DiO is typically added directly to cell monolayers or tissues in a crystalline form<sup>10</sup>, or it is incubated at very high concentrations (~1-20  $\mu$ M) after dilution from a concentration stock solution<sup>11,12</sup>.

Here, the approach was use to the LCNP platform, a multifunctional NP whose inner core is completely hydrophobic and whose surface is simultaneously hydrophilic and amenable to bioconjugation, as a delivery vehicle for DiO. DiO is incorporated into the LCNP core during synthesis, and the NP surface is then functionalized with a PEGylated cholesterol moiety to promote the membrane binding of the DiO-LCNP ensemble to the plasma membrane. This approach resulted in a delivery system that partitioned the DiO into the plasma membrane with greater fidelity and membrane residence time than the free form of DiO delivered from bulk solution (DiO<sub>free</sub>). Further, this method showed that the LCNP-mediated delivery of DiO substantially modulates and drives the rate of specific partitioning of the dye into the lipophilic plasma membrane bilayer. This is achieved while concomitantly reducing the cytotoxicity of the free drug by ~40% by delivering it as an LCNP formulation.

It is anticipated that the methodology described herein will be a powerful enabling technique for researchers whose work involves or requires the cellular delivery of highly hydrophobic cargos that are sparingly soluble or completely insoluble in aqueous solution.

## **PROTOCOL:**

### **1. Preparation of DiO-LCNP and DiO-LCNP-PEG-Chol**

1.1) Dissolve liquid crystalline diacrylate cross-linking agent (DACTP11, 45 mg), 3,3'-dioctadecyloxacarbocyanine perchlorate (DiO, 2 mg), and a free radical initiator (azobisisobutyronitrile, 1 mg) for polymerization in 2 mL of chloroform. Add this to an aqueous solution of acrylate-functionalized surfactant (AC10COONa, 13 mg in 7 mL).

1.2) Stir the mixture for 1 h and sonicate at 80% amplitude for 5 min to produce a miniemulsion consisting of small droplets of the organic material surrounded by polymerizable surfactant in water.

1.3) Heat the mixture to 64 °C in an oil bath to initiate the polymerization of both the cross-linking agent and surfactant as the chloroform slowly evaporates, leaving a DiO-containing NP suspension that is stabilized by the surfactant.

1.4) Filter the NP suspension (3 times) through a 0.2-µm syringe filter to remove any aggregation. Store the filtered NP solution at 4 °C until further use.

#### 1.5) Conjugation of PEG-Chol to DiO-LCNP via EDC coupling.

1.5.1) Dissolve Chol-PEG-NH<sub>2</sub>.HCl (PEG-Chol, 0.9 mM) in 25 mM HEPES buffer (pH 7.0).

1.5.2) Prepare a working solution containing *N*-hydroxy-sulfosuccinimide sodium salt (NHSS, 40 mM) and 1-ethyl-3-(3-(dimethylamino)-propyl) carbodiimide hydrochloride (EDC, 400 mM) in HEPES buffer from concentrated stock solutions.

1.5.3) Immediately add 20 µL of the freshly-prepared working solution of NHSS/EDC to 1.0 mL of DiO-LCNP in HEPES buffer and stir for 5 min.

1.5.4) Add 20 µL of stock solution of Chol-PEG-NH<sub>2</sub>.HCl to this mixture and stir for 2 h.

1.5.5) Briefly centrifuge the reaction mixture at maximum speed (~2,000 x g) for 30 s using a tabletop mini centrifuge and pass the supernatant through a PD-10 size exclusion chromatography column<sup>13</sup> equilibrated with Dulbecco's phosphate-buffered saline (DPBS, 0.1X).

## 2. Characterization of DiO-LCNP and DiO-LCNP-PEG-Chol

2.1) Confirm the successful covalent conjugation of PEG-Chol to the DiO-LCNP surface by gel electrophoresis.

2.1.1) Dissolve 0.5 g of agarose in 50 mL (1x) of tris-borate electrophoresis (TBE; 89 mM Tris (pH 7.6), 89 mM boric acid, and 2 mM EDTA) buffer. Heat the solution in a microwave oven to dissolve the agarose. Allow the solution to cool slightly and pour the contents into a gel plate in the electrophoresis box. Insert a gel comb to create sample wells.

2.1.2) Once the gel has solidified, add the required amount (enough to submerge the gel in the chamber) of TBE running buffer to the chamber.

2.1.3) Add 35 µL of DiO-LCNP sample (amended with glycerol, 5% v/v) to the wells of the gel and run for 20 min at a voltage of 110 V.

2.1.4) Image the gel using a gel imaging system with excitation and emission filters at 488 nm and 500-550 nm, respectively.

2.2) Assess particle size and distribution by dynamic light scattering (DLS) by diluting the DiO-LCNP solution (~200-fold dilution) in PBS (pH ~8, 0.1X) and measuring on a DLS instrument<sup>14</sup>. Measure the zeta-potential of the DiO-LCNPs using an appropriate zeta potential measurement instrument.

### **3. Preparation of Cell Culture Dishes for Delivery Experiments and Imaging**

NOTE: DiO-LCNP labeling is performed on HEK 293T/17 human embryonic kidney cells (between passages 5 and 15) that are cultured as described previously<sup>4</sup>. Perform the delivery experiments and the subsequent cell imaging as described below.

3.1) Prepare 35-mm diameter tissue culture dishes (fitted with 14-mm No. 1 coverglass inserts) by coating them with bovine fibronectin (~100  $\mu$ L at a concentration of 20  $\mu$ g/mL) in DPBS for 2 h at 37 °C.

3.2) Remove the fibronectin coating solution from the culture dish. Harvest HEK 293 T1/7 cells from the T-25 flask by first washing the cell monolayer with 3 mL of DPBS and then by adding 2 mL of trypsin-EDTA (0.5% trypsin-0.25% EDTA).

3.3) Incubate the flask at 37 °C for ~3 min. Remove the trypsin-EDTA and return the flask to the incubator. Once cells are detached from the flask, neutralize the trypsin by adding 4 mL of complete medium (Dulbecco's Modified Eagle Medium; DMEM; amended to contain 10% fetal bovine serum, 5% sodium pyruvate, and 5% antibiotic/antimycotic) to the flask. Determine the cell concentration in the suspension by counting them in a cell counter.

3.4) Adjust the cell concentration to  $\sim 8 \times 10^4$  cells/mL with growth medium. Add 3 mL of the cell suspension to the dish and culture in the incubator overnight; the next day, the cells should be at ~70% confluency and should be ready for use. Adequate cell density is critical for robust and efficient labeling of a high percentage of cells.

### **4. Cellular Delivery of DiO and DiO-LCNPs and Imaging of Fixed Cells**

4.1) Prepare 1-mL solutions of DiO, DiO-LCNP, and DiO-LCNP-PEG-Chol in delivery medium (HEPES-DMEM; DMEM containing 25 mM HEPES) by diluting stock solutions of DiO<sub>free</sub> and DiO-LCNPs; appropriate DiO concentrations for incubation on cells will be ~1-10  $\mu$ M (expressed as either the concentration of DiO<sub>free</sub> or DiO in the form of DiO-LCNP).

4.2) Remove the growth medium from the culture dishes using a serological pipette and wash the cell monolayers (see step 3) two times with HEPES-DMEM (2 mL each wash). Perform the washes by gently adding and removing HEPES-DMEM using a pipette to/from the edge of the dishes.

4.3) Add 0.2 mL of the prepared DiO<sub>free</sub> or DiO-LCNP delivery solutions to the center of the culture dishes and return the dishes to the incubator for an appropriate period of time (typically 15 or 30 min, depending on the needs of the experiment). Longer incubation times add to more nonspecific cellular labeling of non-membranous areas (e.g., cytosol).

4.4) After the incubation period, remove the delivery solutions using a serological pipette. Wash the cell monolayers two times with DPBS (2 mL each wash). Perform the washes by gently adding and removing DPBS to/from the edge of the dish.

4.5) Fix the cell monolayers using 4% paraformaldehyde (prepared in DPBS) for 15 min at room temperature. **Caution:** Paraformaldehyde is flammable, a respiratory irritant, and a suspected carcinogen.

4.6) Remove the paraformaldehyde solution using a pipette and gently wash the cells 1 time with DPBS (2 mL) by adding and removing DPBS using pipette to/from the edge of the dishes.

4.7) The fixed cells are ready to be imaged for the presence of a membranous fluorescence signal using confocal laser scanning microscopy (CLSM). Perform the imaging immediately or, alternatively, replace the medium with DPBS containing 0.05% NaN<sub>3</sub> and store the dishes at 4 °C. **CAUTION!** NaN<sub>3</sub> is toxic; exercise extreme caution when using NaN<sub>3</sub>.

NOTE: Fixed samples stored in this manner should be imaged within 48 h for optimum results.

## 5. Cellular Delivery of DiO and DiO-LCNPs and FRET Imaging in Live Cells

NOTE: In this method, cells are colabeled with 6 μM each DiO-LCNP-PEG-Chol (where DiO is a FRET donor) and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil<sub>free</sub>, where Dil is a FRET acceptor). The release of DiO from DiO-LCNP-PEG-Chol and its incorporation into the plasma membrane is confirmed by an observed increase in energy transfer from the DiO donor to the Dil acceptor.

5.1) Label cells sequentially with DiO-LCNP-PEG-Chol and Dil<sub>free</sub> using the method described in step 4.

5.2) After staining, wash the cells 1 time with DPBS (2 mL) using a serological pipette and replace the wash buffer with 2 mL of live cell imaging solution (LCIS).

5.3) On a microscope stage equipped with a heated incubation chamber, image the live cell sample using a confocal microscope (60x objective) with a FRET imaging configuration at 30 min intervals over a 4-h period by exciting the DiO donor at 488 nm and collecting full emission spectra of both the DiO donor and the Dil acceptor from 490-700 nm with a 32-channel spectral detector.

NOTE: For more information on FRET imaging, please see Reference 15.



5.4) Determine the time-resolved emission intensity of both the DiO donor and the DiI acceptor from cells stained with DiO-LCNP-PEG-Chol and DiI. Calculate the time-resolved acceptor/donor FRET ratio ( $\text{DiI}_{\text{emi}}/\text{DiO}_{\text{emi}}$ ) for the images, which will steadily increase and eventually plateau once the amount of DiO donor partitioned into the membrane has reached a maximum<sup>16</sup>.

## 6. Cytotoxicity Assay of DiO and DiO-LCNPs to the HEK 293T/17 Cells

NOTE: The cytotoxicity of the DiO-LCNP materials is assessed using a tetrazolium dye-based proliferation assay<sup>17</sup>. Cells are cultured in a multiwell plate in the presence of varying concentrations of the materials under conditions that emulate delivery/labeling. The cells are then cultured for 72 h to allow for proliferation. A dye (MTS, (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) is then added to the wells, and metabolically active cells convert the dye into a blue formazan product. The amount of color formation is directly proportional to the number of viable cells.

6.1) Harvest HEK 293 T1/7 cells from the T-25 flask by following the procedure described in step 3.2. Seed HEK 293T/17 cells (5,000 cells/100  $\mu\text{L}$ /well) to the wells of a 96-well tissue culture-treated plate and culture for 24 h.

6.2) Completely remove the culture media from the wells using a micropipette and add 50  $\mu\text{L}$  of HEPES-DMEM containing  $\text{DiO}_{\text{free}}$ , DiO-LCNPs, or DiO-LCNP-PEG-Chol at increasing concentrations to replicate wells. Incubate on the cells at 37 °C for 30 min.

NOTE: Typically, replicates done in triplicate or quadruplicate are sufficient to yield statistically-reliable data.

6.2.1) After incubation, remove the delivery medium containing the materials using a micropipette and replace it with 100  $\mu\text{L}$  of growth medium. Culture the cells for 72 h.

6.3) Add 20  $\mu\text{L}$  of the tetrazolium substrate to each well, return the plate to the incubator, and allow color formation to proceed at 37 °C for 4 h. Read the absorbance (abs) of the formazan product at 570 nm (absorption minima for the DiO-LCNPs used in this study) and 650 nm (for subtraction of nonspecific background absorbance) using a microtiter plate reader.

6.4) Plot the differential absorbance value ( $\text{abs}_{570} - \text{abs}_{650}$ ) versus material concentration and report the results as percent of control cell proliferation (degree of proliferation of cells in cell culture medium only).

## 7. Data Analysis.

7.1) Statistically analyze the data with a univariate analysis of variance (ANOVA). For multiple comparisons, apply the Bonferroni's post hoc test. Provide all average values as  $\pm$  standard error of mean (SEM) unless otherwise mentioned. NOTE: The acceptable probability for significance was  $p < 0.05$ .

## REPRESENTATIVE RESULTS:

LCNPs were prepared in which the hydrophobic core of the NP was loaded with a representative membrane-labeling probe to demonstrate the utility of the LCNP as an efficient delivery vehicle for hydrophobic cargos. For this purpose, the cargo chosen was the highly water-insoluble potentiometric membrane-labeling dye, DiO. DiO-loaded LCNPs (DiO-LCNPs) were synthesized using a two-phase mini-emulsion technique with the chemical components DACTP11, AC10COONa, and DiO, as shown in **Figure 1**<sup>18</sup>. In this NP system, the covalently-linked polymeric network of the crystalline cross-linking agent DACTP11 provides a stable hydrophobic core where the DiO resides within the interstitial spaces in the crosslinked network. The carboxylate groups on the NP surface provide colloidal stability to the particle in aqueous media while also serving as a functional group “handle” for the attachment of cell-targeting ligands (and other biologicals). To tether the DiO-LCNPs to the plasma membrane, an amine-terminated PEGylated cholesterol moiety (PEG-Chol) was covalently attached to the LCNP surface *via* EDC coupling (**Figure 1**). After NP synthesis and the confirmation of successful bioconjugation, the ability of the DiO-LCNPs to label the plasma membrane of living cells and to deliver the embedded DiO cargo to the lipophilic portion of the plasma membrane bilayer with improved specificity and kinetics compared to the free form (DiO<sub>free</sub>) delivered from bulk solution was assessed.

As shown in **Figure 2A**, DiO<sub>free</sub> (6.0  $\mu$ M) robustly stained the plasma membrane with high efficiency (nearly 100% of cells labeled) after 15 min of incubation with HEK 293T/17 cells. However, significant cellular internalization of DiO<sub>free</sub> was observed upon only a modest increase in the incubation time to 30 min (**Figure 2A and C**). The extent of DiO<sub>free</sub> internalization was determined by time-resolved colocalization experiments in which cells were first incubated with 6.0  $\mu$ M DiO<sub>free</sub> (30 min). The DiO<sub>free</sub>-containing incubation medium was then removed, and the cells were subsequently cultured for up to 4 h. The plasma membranes of the cells were counterstained with a dye-labeled membrane phospholipid (phosphoethanolamine conjugated to Rhodamine B; PE-Rhoda). As shown in **Figure 2C**, after 15 min of incubation, almost 100% of DiO<sub>free</sub> was colocalized with the PE-Rhoda marker (Pearson's colocalization coefficient; PCC =  $0.99 \pm 0.01$ ). However, after 30 min of incubation ~80% of DiO<sub>free</sub> remained bound to the membrane (~20% internalized). The degree of DiO<sub>free</sub> internalization increased steadily as the incubation time was extended to as long as 4 h, and this was reflected in the concomitant decrease in the PCC between the DiO and Rhoda-PE (**Figure 2C**). A fit of the data to a one-phase exponential decay equation revealed that the DiO<sub>free</sub> internalization reached a maximum of ~50% at 1 h, with an internalization rate ( $k = 0.045 \text{ min}^{-1}$ ) and half-life (15 min) that reflected the rapid and efficient cellular uptake of DiO<sub>free</sub>. These data demonstrate the rapid time-dependent transition of DiO<sub>free</sub> from the plasma membrane to the cytosol, where it remained largely excluded from the nucleus over the 4 h time period examined.

Given the uncontrolled cellular uptake of DiO<sub>free</sub>, the goal became to modulate this behavior through the delivery of DiO as an LCNP-PEG-Chol NP formulation. When delivered as an LCNP-PEG-Chol bioconjugate, a more persistent membrane residence

time of the DiO compared to DiO<sub>free</sub> was noted (**Figure 2B**). After 15 min of incubation of the DiO-LCNP-PEG-Chol with the cells, nearly 100% of the DiO signal was located at the plasma membrane, where it was colocalized with the PE-Rhoda membrane marker, a result that was comparable to that observed for DiO<sub>free</sub>. It was noted, however, that while the DiO<sub>free</sub> labeling of the membrane was quite uniform and contiguous, the staining pattern of the DiO-LCNP-PEG-Chol after 15 min of incubation was more punctate and not nearly as uniform in nature (**Figure 2B**). This result indicated that the DiO-LCNP-PEG-Chol NPs were collecting in discrete regions within the plasma membrane. When the incubation time was increased to 30 min, ~94% of the DiO signal remained at the membrane (compared to ~80% for DiO<sub>free</sub> at this same time point) (**Figure 2B and C**). This trend became even more pronounced as the cells were cultured with the DiO-LCNP-PEG-Chol NPs for increasingly longer times after the initial 30 min incubation. For example, after culture for 1 h after the initial incubation, nearly 80% of the DiO-LCNP-PEG-Chol NP signal remained membranous and colocalized with the PE-Rhoda marker (compared to ~55% for DiO<sub>free</sub>). Notably, the cellular internalization of the DiO-LCNP-PEG-Chol NPs reached a maximum of 30% at 2 h (70% membrane retention). This corresponds to a cellular uptake rate ( $k = 0.024 \text{ min}^{-1}$ ; half-life = 29 min) that is exactly one half of that observed for the internalization of DiO<sub>free</sub>.

Next, the ability of the NP-embedded DiO cargo to efficiently efflux out of the LCNP core and enter the lipophilic environment of the plasma membrane bilayer in a controlled and time-dependent manner was determined. To assess this, a FRET-based strategy was devised wherein the DiO serves as a FRET donor engaged in energy transfer to the acceptor dye, Dil. As expected, upon initial labeling ( $t = 0 \text{ min}$ ), the emission signal was dominated by the DiO donor contained within the membrane-tethered NPs (**Figure 3B**). FRET imaging of this same field 4 h later ( $t = 240 \text{ min}$ ), however, revealed a significant increase in the emission signal of the Dil acceptor, providing strong evidence of the transition of the DiO donor from the LCNP core into the plasma membrane bilayer (**Figure 3C**). Examination of the time-resolved nature of this transition showed a steady decrease in DiO donor emission coupled with a corresponding increase in Dil acceptor emission over the 4-h experimental window (**Figure 3D**). Notably, the FRET efficiency during this transition reached its maximum at ~180 min post-initial labeling, suggesting that the efflux and membrane partitioning of the DiO donor had reached its maximum at this time point (**Figure 3E**). These data provided strong evidence of the time-resolved partitioning of the DiO from the NP to the plasma membrane bilayer that reached its maximum ~3 h post-initial labeling with the DiO-LCNP-PEG-Chol NPs.

Finally, comparative cytotoxicity analysis was performed for DiO<sub>free</sub> versus DiO-LCNP-PEG-Chol. When incubated with HEK 293T/17 cells (at a DiO concentration of 6  $\mu\text{M}$  in both cases), it was clear that the encapsulation of the DiO within the LCNP core attenuated its cytotoxicity. While DiO<sub>free</sub> elicited cellular viabilities of ~50%, cells incubated with DiO-LCNP-PEG-Chol exhibited cellular viabilities ~90%. (**Figure 4**). Cumulatively, the cell labeling data coupled with the cellular toxicity data demonstrate enhancements in both the efficiency of DiO-based membrane labeling and the modulation of the cytotoxicity of DiO.

## FIGURE LEGENDS:

**Figure 1: Schematic representation of membrane-binding DiO-LCNP-PEG-Chol.** DiO-LCNP is composed of an acrylate liquid crystal cross-linking agent (DACTP11); a carboxyl-terminated polymerizable surfactant (AC10COONa); and a lipophilic dye, DiO. Cholesterol-terminated poly(ethylene glycol) (PEG-Chol) was conjugated to DiO-LCNP *via* EDC coupling. Addition of Chol to the DiO-LCNP surface mediates preferential binding of the NP to the plasma membrane.

**Figure 2: Time-resolved cellular uptake of DiO<sub>free</sub> in HEK293T/17 cells.** DiO<sub>free</sub> (6.0  $\mu$ M, panel A) or DiO-LCNP-PEG-Chol ([DiO] = 6.0  $\mu$ M, panel B) was incubated on cell monolayers for 15 min, removed, and replaced with growth medium; the cells were cultured for the times indicated (up to 4 h). Cells were subsequently stained with PE-Rhoda (2.0  $\mu$ M) and fixed. The samples were imaged for DiO (green) and membrane-bound PE-Rhoda (red) using CLSM. (C) Time-resolved plot of the percent of membrane-bound DiO<sub>free</sub> or DiO-LCNP-PEG-Chol signal as a function of increasing incubation time. The data was obtained from the Pearson's colocalization coefficient (PCC,  $n = 3 \pm$  standard deviation) of the green (DiO) and red (PE-Rhoda) channels, and is expressed as a percentage ( $\pm$  SEM) after normalization to the PCC corresponding to 15 min of incubation. The scale bar is 20  $\mu$ m. Reprinted with permission from Reference 18.

**Figure 3: Time-resolved FRET confirms the efflux of DiO from DiO-LCNP-PEG-Chol to the plasma membrane.** HEK293T/17 cells were costained with DiO-LCNP-PEG-Chol and Dil, where the DiO (green emitting) and Dil (red emitting) dyes act as the FRET donor and acceptor, respectively. (A) DIC image of the live cells costained with DiO-LCNP-PEG-Chol ([DiO] = 6.0  $\mu$ M) and Dil (6.0  $\mu$ M). The sample was imaged to monitor the change in FRET signal over the period of 4 h. (B) and (C) are the FRET images of the live cell on the same focal plane at  $t = 0$  min and  $t = 240$  min, respectively. (D) Normalized, time-resolved emission intensity of the DiO donor and Dil acceptor from cells stained with DiO-LCNP-PEG-Chol and Dil and imaged in FRET excitation mode. (E) Time-resolved FRET ratio ( $Dil_{emi}/DiO_{emi}$ ) for cells labeled with DiO-LCNP-PEG-Chol and Dil and imaged in FRET configuration. The scale bar is 20  $\mu$ m. Reprinted with permission from Reference 18.

**Figure 4: Quantification of cytotoxicity.** DiO<sub>free</sub>, LCNP, DiO-LCNPs, or DiO-LCNP-PEG-Chol were incubated on HEK 293T/17 cell monolayers for 15 min and then removed. Cells were washed and cultured in growth medium for 72 h prior to MTS assay. The bar graph represents a comparison of the cell viability ( $n = 5 \pm$  SEM) of DiO<sub>free</sub>, LCNP, DiO-LCNP, and DiO-LCNP-PEG-Chol at [DiO] = 6.0  $\mu$ M. The difference between DiO<sub>free</sub> and LCNP, DiO-LCNP, or DiO-LCNP-PEG-Chol are significant ( $p < 0.001$ ) at 72 h of incubation. Reprinted with permission from Reference 18. The data was analyzed using the univariate analysis of variance (ANOVA); the Bonferroni's post hoc test was used for multiple comparisons.

## DISCUSSION:

A continuing goal of NMDD is the controlled targeting and delivery of drug formulations to cells and tissues, combined with simultaneous improved drug efficacy. One specific class of drug molecules for which this has posed a significant challenge is hydrophobic drugs/imaging agents that have sparingly to no solubility in aqueous media. This problem has plagued the transition of potent drugs from *in vitro* cell culture systems to the clinical setting and has resulted in a number of promising drug molecules being “shelved” or abandoned and not pursued further in the clinical setting. Wortmannin (Wtmn), for example, is a potent inhibitor of phosphatidylinositol 3' kinases and phosphatidylinositol 3' kinase-related kinases and has great potential as an anticancer agent, but its lack of water solubility has hampered its progression in clinical trials.

Recently, Karve *et al.* showed improved water solubility of Wtmn when formulated as a lipid-polymer NP assembly<sup>19</sup>. One arena that could greatly benefit from this type of improved, controlled delivery as an NP formulation is the delivery of hydrophobic drugs and imaging agents to the plasma membrane. Thus, the goal here was to address this technical hurdle and to develop a methodology to overcome this technological hurdle.

In developing this methodology, a model membrane-targeting dye, DiO, was selected that has historically been plagued by poor solubility in aqueous media. This imparts significant challenges in the specific delivery of the dye to the plasma membrane. Among these challenges are the need to add the crystalline form of the dye directly to cells or tissues<sup>10</sup> or to incubate cells with very high concentrations of DiO after dilution from stock solutions containing solvents such as DMSO<sup>11,12</sup>. The approach here was two-fold: 1) incorporate the DiO into the hydrophobic core of LCNPs during NP synthesis and 2) covalently modify the as-synthesized DiO-LCNPs with a PEGylated cholesterol conjugate (DiO-LCNP-PEG-Chol) to facilitate the direct interaction of the DiO-loaded NPs with the plasma membrane. Indeed, this approach effectively improved a number of aspects of the delivery of DiO to the plasma membrane.

First, the membrane residence time of DiO was effectively doubled when delivered as a LCNP formulation compared to when DiO was delivered free from bulk solution. This extended membrane residence time was attributed to the localization of the DiO-LCNP-PEG-Chol conjugates to the lipid raft microdomains of the plasma membrane, as confirmed by co-staining experiments. Second, the partitioning of the DiO cargo into the lipid bilayer was confirmed by FRET experiments in which the DiO served as the donor to a membrane-resident Dil acceptor. Finally, the slow, sustained release of the DiO cargo into the membrane bilayer effectively attenuated the cytotoxicity of the DiO by ~40%.

It is important to highlight several points in the protocol that are critical for success. First, the percent loading of the DiO into the LCNP core must be empirically optimized in trial synthesis runs to obtain a balance between the generation of high-quality DiO-LCNP-PEG-Chol NPs and the desired level of fluorescence signal in cellular delivery experiments. Likewise, the bioconjugation of the Chol-PEG-NH<sub>2</sub> moiety to the DiO-LCNP surface must be optimized to obtain the desired level of cellular labeling. Finally,

the number of cells applied to the fibronectin-coated culture dishes is critical for success, and care must be taken to seed the cells at a density of  $\sim 8 \times 10^4$  cells/mL ( $\sim 2.4 \times 10^5$  cells per dish). When the cells are seeded too sparsely, it results in inefficient membrane labeling, while seeding the cells too densely results in the acquisition of poor images that do not clearly show membranous labeling.

One potential limitation of the as-described protocol is that the efficiency of incorporation of various other membrane-targeting dyes and drugs will vary depending on the hydrophobicity of the dye/drug cargo. In these instances, the LCNP synthesis protocol will have to be tested empirically to determine the optimum synthesis conditions for ideal dye/drug cargo incorporation.

In summary, a method has been developed for the LCNP-based controlled delivery of a hydrophobic dye cargo to the plasma membrane of living cells that overcomes many of the technical challenges associated with the cellular delivery of water-insoluble cargos. The overall significance of the method is that it clearly improves the specific delivery of cargo to the plasma membrane while simultaneously attenuating the concomitant cytotoxicity that is associated with the delivery of hydrophobic cargos at high concentrations from bulk solution. This approach should find wide utility in the delivery of similarly-challenging hydrophobic dye/imaging agent cargos and will likely help to facilitate the transition of effective, yet poorly water-soluble, cargos from the experimental regime to the clinical setting.

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#### **DISCLOSURES:**

The authors declare that they have no competing financial interests.

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Figure 1

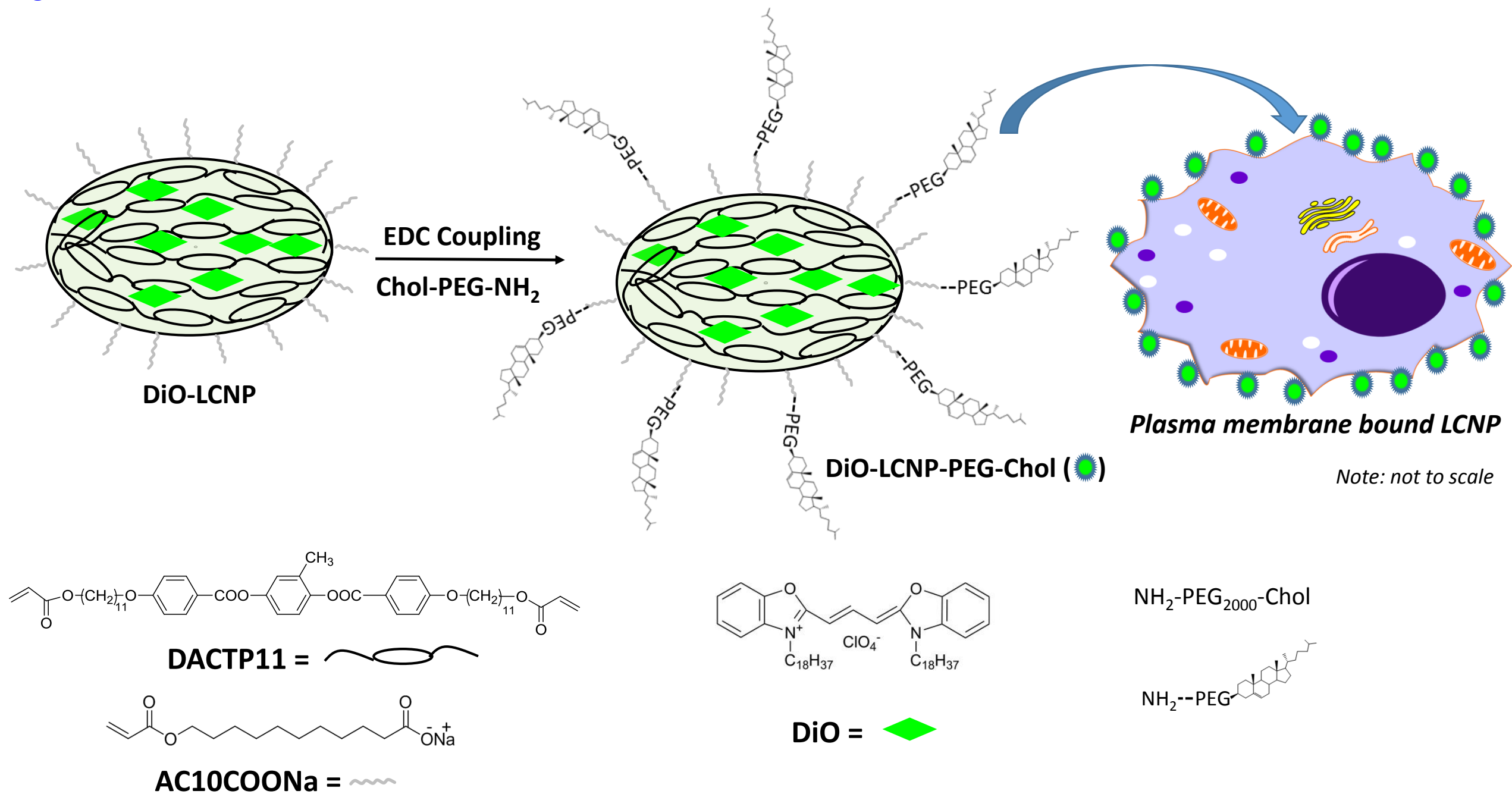




Figure 2

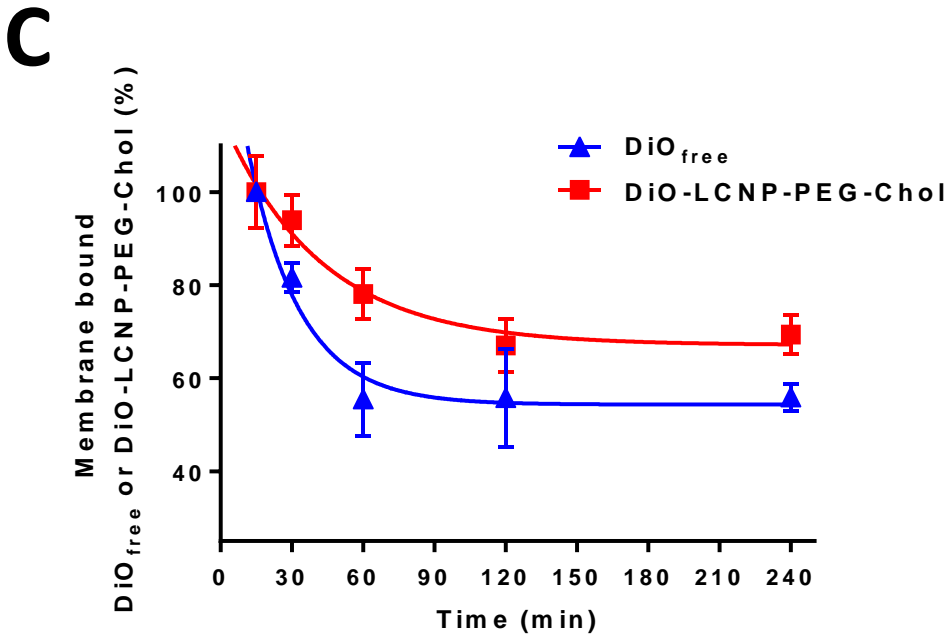
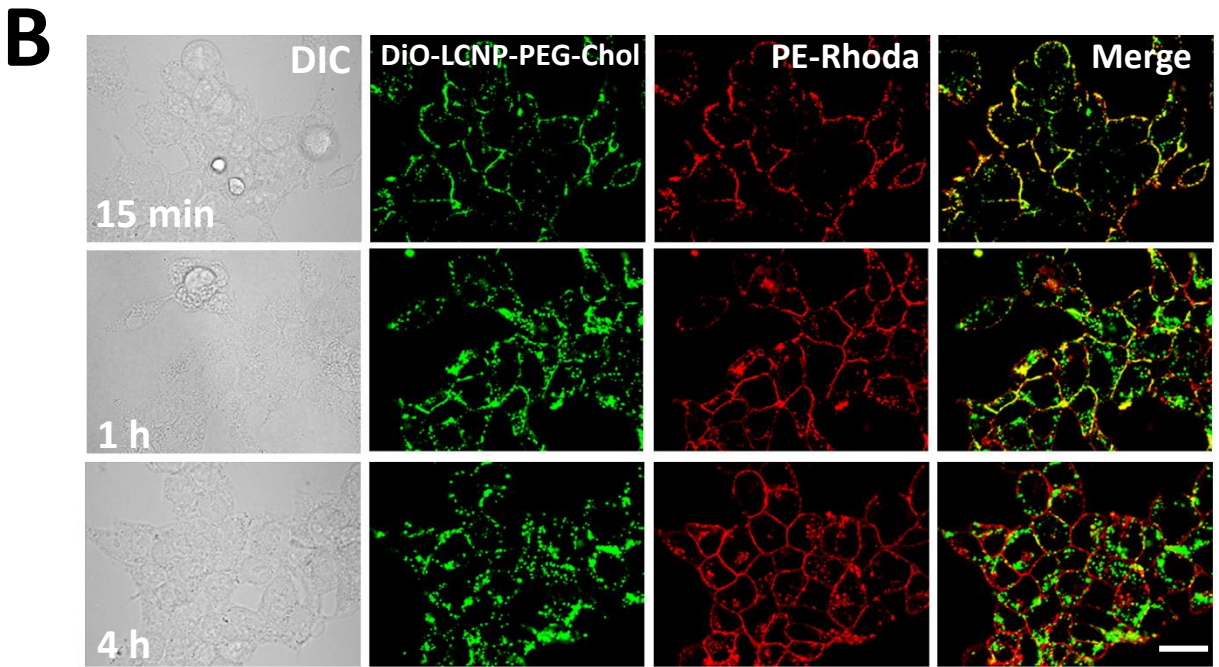
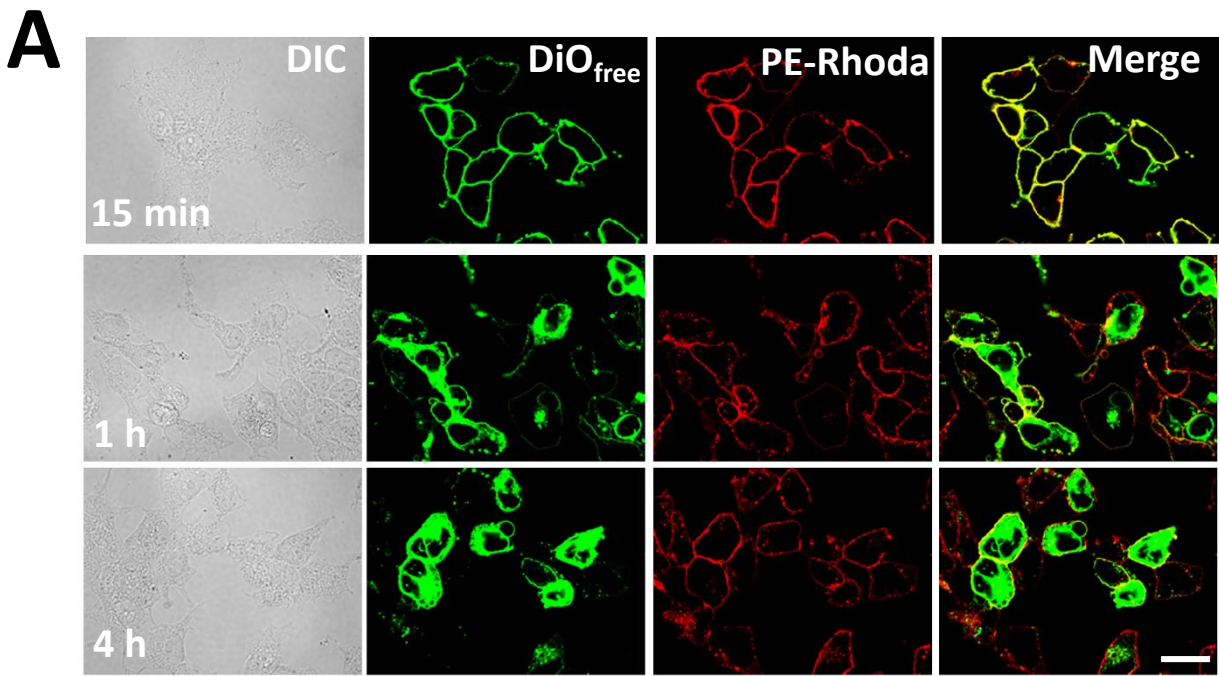


Figure 3

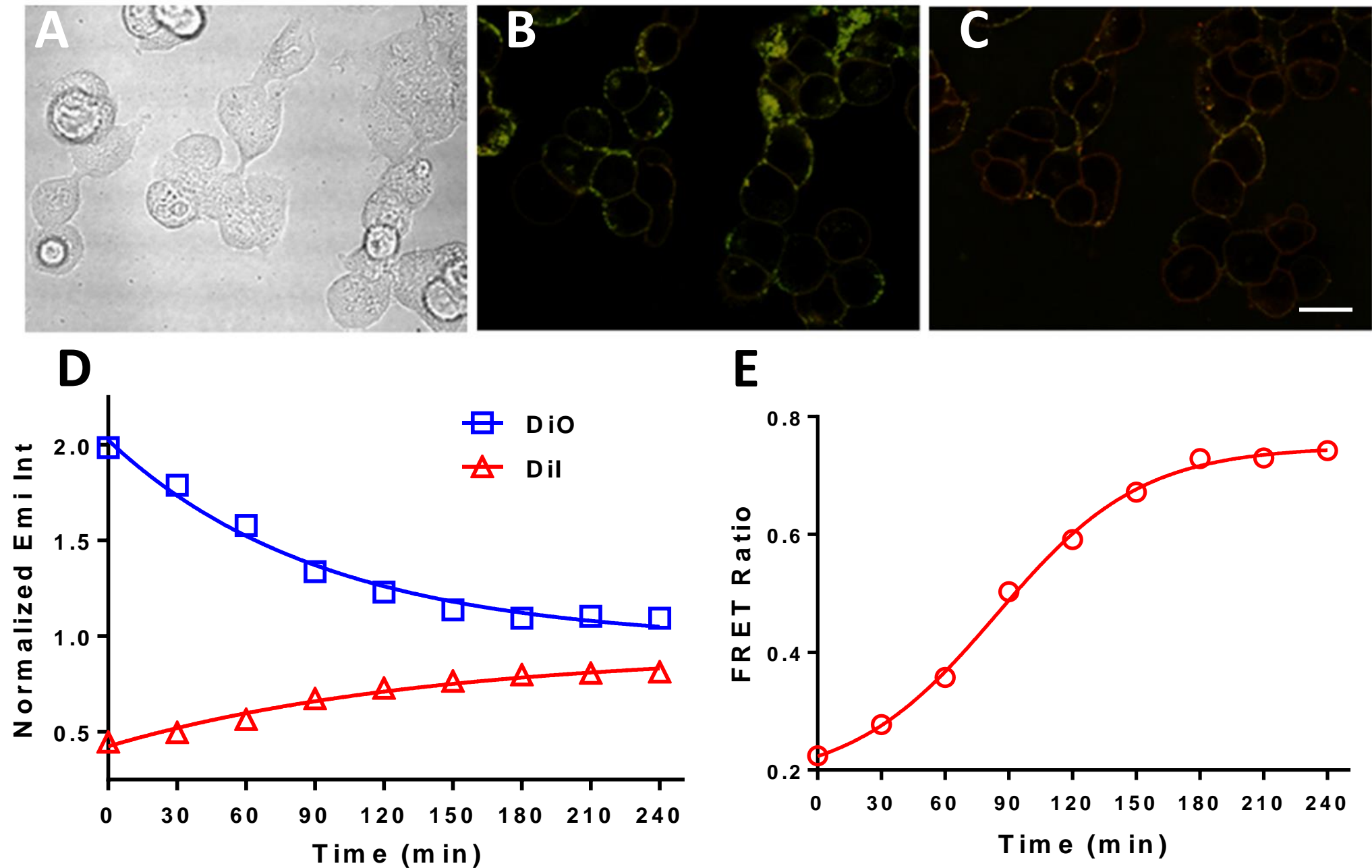
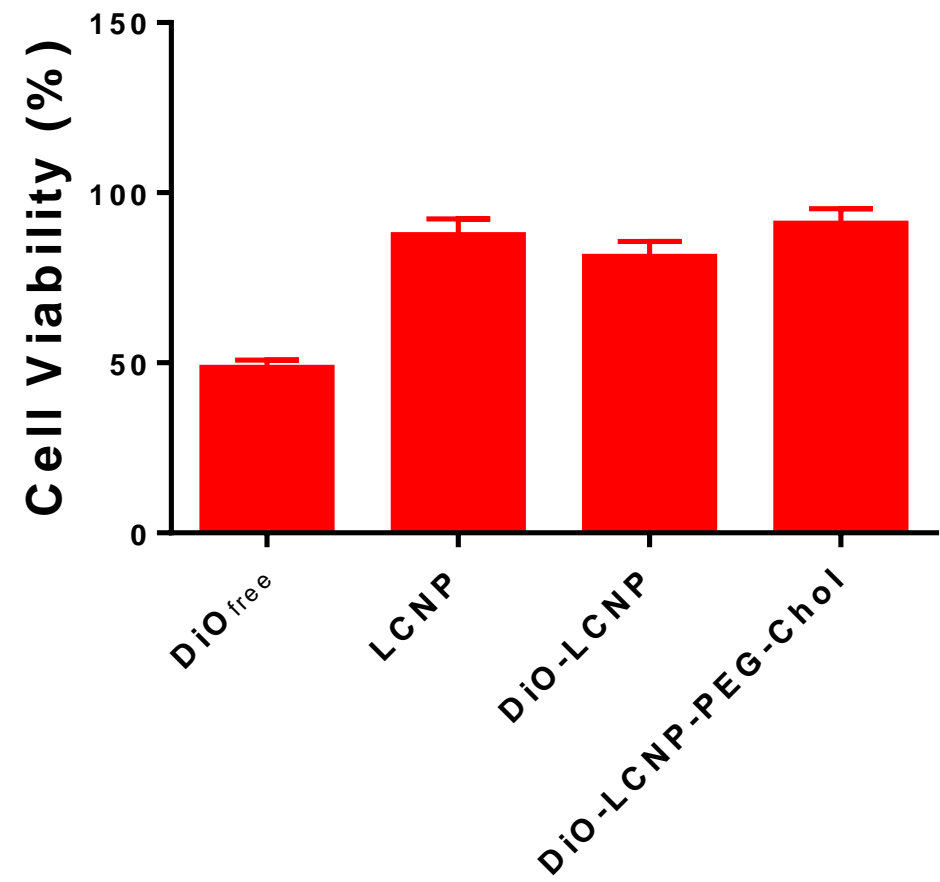


Figure 4



**Material/Equipment**

1-ethyl-3-(3-(dimethylamino)-propyl)carbodiimide hydrochloride (EDCA)  
3,3'-dioctadecyloxacarbocyanine perchlorate (DiO)  
Cholesterol poly(ethylene glycol) amine hydrochloride  
Countess automated cell counter  
Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (Dil)  
Dulbecco's Modified Eagle's Medium (DMEM)  
Dulbecco's Phosphate Buffered Saline (DPBS)  
Dynamic light scattering instrument  
Fibronectin Bovine Protein, Plasma  
Formaldehyde (16%, W/V)  
Human embryonic kidney cells (HEK 293T/17)  
Live cell imaging solution (LCIS)  
MatTek 14 mm # 1.0 coverglass insert cell culture dish  
Modified Eagle Medium (DMEM) containing 25 mM HEPES  
N-hydroxysulfosuccinimide sodium salt (NHSS)  
Nikon A1si spectral confocal microscope  
Trypan Blue Stain (0.4%)  
Zeta potential instrument  
Ultrasonic Processor  
Mini Cetntrifuge  
PD-10 Sephadex™ G-25 Medium  
Bio-Rad ChemiDoc XRS Imaging System  
Trypsin-EDTA (0.25%), phenol red

<b>Company</b>	<b>Catalog Number</b>
ThermoFisher	E2247
Sigma Aldrich	D4292-20MG
Nanocs, Inc.	PG2-AMCS-2k
ThermoFisher	C10227
Sigma Aldrich	468495-100MG
ThermoFisher	21063045
ThermoFisher	14040182
ZetaSizer NanoSeries (Malvern Instruments Ltd., Worcestershire, UK)	
ThermoFisher	33010018
ThermoFisher	28906
American Type Culture Collection	ATCC® CRL-11268™
ThermoFisher	A14291DJ
MatTek corporation	P35G-1.0-14-C
ThermoFisher	21063045
ThermoFisher	24510
Nikon Instruments	
ThermoFisher	T10282
ZetaSizer NanoSeries (Malvern Instruments Ltd., Worcestershire, UK)	
Sonics and Materials Inc	GEX 600-5
Benchmark	Mini-fuge-04477
GE Healthcare	17-0851-01
Bio-RAD	76S/07434
ThermoFisher	25200056

## **Comments/Description**

Hazardous/ make stock solution in DMSO

Hazardous/ make stock solution in DMSO

Warm in 37°C before use

Warm in 37°C before use

Make stock solution 1mg/mL using DPBS. Use 20-30 µg/mL for coating MetTek dish, 2 h@ 37°C

Hazardous, dilute to 4% using DPBS

Warm in 37°C before use

Warm in 37°C before use

mix as a 50% to the cell suspension before counting the cells







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US Naval Research Laboratory  
4555 Overlook Avenue SW  
Washington, DC 20375

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Senior Science Editor  
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Cambridge, MA 02140

Dear Dr. Singh:

Thank you for your consideration of our submitted manuscript “Targeted Plasma Membrane Delivery of a Hydrophobic Cargo Encapsulated in a Liquid Crystal Nanoparticle Carrier” for publication in *Journal of Visualized Experiments* and for the thorough and thoughtful comments. Attached is a revised version of the manuscript (55181\_R2\_2016\_09\_20.docx) that addresses all of the editorial concerns/reviewer issues raised. Any changes to the manuscript are highlighted as mentioned in the response below. Below is a detailed, by-line item response to each of the critiques raised during the review process. Also attached are revised figures (as .pdf file).

Thank you for your interest in our work and for your consideration for publication of our manuscript in JoVE.

Sincerely,



James B. Delehanty, Ph.D.  
ph: (202) 767-0291  
email: [james.delehanty@nrl.navy.mil](mailto:james.delehanty@nrl.navy.mil)

Editorial comments:

Response: Any changes made by the authors are highlighted in green in the revised version of the manuscript.

- NOTE: Please download this version of the Microsoft word document (File name: 55181\_R1\_072616) for any subsequent changes. Please keep in mind that some editorial changes have been made prior to peer review.

- Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

- Formatting: NaN3 is toxic and requires a caution statement.

Response: This has been added to Protocol section 4.7.

- Length exceeds 3 pg; 2.75 pg or less (1 pg minimum) must be highlighted for filming. Steps involving calculations, such as 5.4 & 6.4, should not be highlighted.

Response: ~2.75 of the ~ 4 pgs of protocol text have been highlighted in yellow for filming.

- Please copyedit the manuscript for many grammatical errors. Such editing is required prior to acceptance. Some examples are indicated below:

-5.7 – “The fixed are ready”; “Perform the immediately”; also, please use imperative tense or convert to a note.

-Section 6 note – “is assess”

-Line 471 – “of aspects DiO delivery”

-Line 475 – “was attributed this”

Response: The manuscript has been copy-edited and the appropriate changes have been highlighted in green.

- Additional detail is required:

-2.1.1 – What is the composition of TBE?

-3.3 – What is the composition of complete medium?

Response: The details have been added to Protocol sections 2 and 3.

- Branding: Line 98 – Pluronic®

Response: Pluronic® has been replaced with the chemical name “Poloxamers”.

- Results: Figure 4 – Please indicate the statistical test used.

Response: The data analysis method has been added to section 7.

- Discussion: Please discuss the limitations of the protocol.

Response: Lines 496-500, we have added language that discusses the potential challenge of incorporating other dye and drug cargos that might vary in their degree of hydrophobicity. In these instances, different formulations and ratiometric mixes of the dye/drug cargo will have to be empirically tested to determine the optimal mix for successful cargo incorporation into the LCNP.

- If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.

Response: The appropriate statement has been added to the figure legend to figures 2-4.

- JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

Response: DOIs (for those references that have them) have been added and are highlighted green.

**IMPORTANT:** Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

- NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a well written manuscript that is extremely detailed and easy to follow. Having made lipid nanoparticles in the lab before, this is an excellent way to make cross-linked varieties which are more stable.

Major Concerns:

In figure 2 the DIC images are hard to see. Can the contrast be tuned so that the cells appear better in the image?

Response: The DIC image has been tuned so that the cells appear better in the image.

Was a statistical analysis done on figure 4? How relevant is the data?

Response: This information has been added in Protocol section 7.

Minor Concerns:

The green highlighted references are not in the same format as the others.

Response: These references have been fixed.

Additional Comments to Authors:

N/A

Reviewer #2:

Manuscript Summary:

The authors newly produced a liquid crystal NP (LCNP)-based delivery system for the controlled delivery of a water-insoluble dye, 3'-dioctadecyloxacarbocyanine perchlorate (DiO), from within the NP core to the hydrophobic region of plasma membrane bilayer.

Overall this study has clear-cut purpose and was performed by promising techniques, although methodology is simple to some studies previously reported. I think two minor questions should be answered before publication.

Major Concerns:

N/A

Minor Concerns:

1. How to confirm the DiO concentrations for incubation on cells are same expressed as either the concentration of DiOfree or DiO in the form of DiO-LCNP.

Response: The concentration of the DiO in the DiO-LCNP was determined by using the molar extinction coefficient of a known concentration of DiO in organic solvent. DiOfree and DiOLCNP were adjusted to the same DiO concentrations prior to incubation with the cells.

2. Please show the gel electrophoresis results for confirming successful covalent conjugation of PEG-Chol to the DiO-LCNP surface.

Response: Confirmatory gel electrophoresis images are contained in the original manuscript which is referenced in the protocol section.

Additional Comments to Authors:

N/A



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**Supplemental File (as requested by JoVE)**  
Delehanty\_CCC\_permission.pdf

