

Submission ID #: 71788

Scriptwriter Name: Pallavi Sharma

Project Page Link: https://review.jove.com/files_upload.php?src=21895388

Title: Light- and X-ray-Activated Liposomes for Controlled Gene Editing and Drug Delivery

Authors and Affiliations:

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Author Questionnaire

1. **Microscopy:** Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **YES**
5.2.1 — zebrafish microinjection, performed under a stereomicroscope.
Videographer: Please film the scope shot using the scope kit
2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes**
3. **Filming location:** Will the filming need to take place in multiple locations?
4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials.

Current Protocol Length

Number of Steps: 23

Number of Shots: 39

Interviews

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

INTRODUCTION:

~~**REQUIRED:** Summarize your research focus and the main questions it aims to answer.~~

- 1.1. **Yagiz Alp Aksoy:** Our research focuses on developing light- and X-ray-activated capsules for controlled delivery of gene-editing tools and drugs.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the research gap or limitation in existing methods and how this protocol addresses them.**~~

- 1.2. **Yagiz Alp Aksoy:** Conventional gene delivery affects the whole body, whereas this protocol enables localized gene editing by activating delivery only in illuminated cells.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~**What does this protocol allow researchers to study or measure?**~~

- 1.3. **Yagiz Alp Aksoy:** This protocol enables researchers to precisely activate and monitor gene editing in cells and living embryos.
 - 1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~**Describe the most important consideration or challenge when performing this protocol.**~~

- 1.4. **Wei Deng:** The key challenge while performing this protocol is keeping the capsules protected from light until activation, as unintended light exposure can compromise their function.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Wei Deng:** Following the procedure, sequencing, flow cytometry, or fluorescence analysis can be used to confirm successful on-target gene editing.
 - 1.5.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

This research has been approved by the Animal Research Ethics Committee at Macquarie University

Protocol

2. Preparation of Light-Triggered VP-Liposomes for Encapsulation of CRISPR-Cas9 RNP

Demonstrator: Yagiz Aksoy

- 2.1. To begin, prepare the hydration solution by combining the single guide RNA at 0.01 micromolar and the Cas9 (*Cas-Nine*) protein at 0.1 milligrams per milliliter in 500 microliters of nuclease-free water [1]. Pre-incubate the hydration solution at room temperature for 5 minutes to form the ribonucleoprotein, or RNP (*R-N-P*), complex [2].
 - 2.1.1. WIDE: Talent combining the sgRNA and the Cas9 protein in 500 microliters of nuclease-free water.
 - 2.1.2. Talent places the tube with hydration solution in the rack and keeping it aside.
- 2.2. Hydrate the dried lipid film by adding the 500-microliter RNP solution directly onto the film [1-TXT]. Vortex vigorously for 30 seconds, then allow the suspension to stand at room temperature for 30 minutes, with intermittent vortexing every 10 minutes [2].
 - 2.2.1. Talent adding the 500-microliter ribonucleoprotein solution directly onto the dried lipid film. **TXT: Lipid Composition: DOTAP, DOPE, Cholesterol, and Verteporfin**
 - 2.2.2. Talent vortexing the hydrated lipid suspension and keeping it aside.
- 2.3. Then, extrude the multilamellar suspension 11 times through a 200-nanometer polycarbonate membrane using a mini-extruder to produce unilamellar liposomes of uniform size [1].
 - 2.3.1. Talent extruding the multilamellar suspension through a 200-nanometer polycarbonate membrane using a mini-extruder.
- 2.4. Store the verteporfin, or VP (*V-P*), liposomes at 4 degrees Celsius, protected from light, and use them within 48 hours of preparation for optimal activity [1].
 - 2.4.1. Talent placing the VP-liposomes into storage protected from light at 4 degrees Celsius.

3. Physicochemical Characterization of VP-Liposomes

- 3.1. Dilute the liposome suspension at a 1 to 100 ratio in nuclease-free water [1]. Measure the hydrodynamic diameter, the polydispersity index, and the zeta potential by dynamic light scattering [2]. Perform all the dynamic light scattering measurements in triplicate and report the values as the mean plus or minus the standard deviation [3].
 - 3.1.1. Talent diluting the liposome suspension at a 1:100 ratio in nuclease-free water.

3.1.2. Talent placing the diluted liposome suspension into the dynamic light scattering instrument for measurement.

3.1.3. LAB MEDIA: 71788_shot_3.1.3_DLS_results.

3.2. Deposit 10 microliters of the diluted liposome suspension onto a carbon-coated copper grid for transmission electron microscopy [1]. Stain the sample with 2 percent uranyl acetate for 1 minute [2]. Image the sample at 100 kilovolts [3] and acquire at least 10 representative images per batch [4].

3.2.1. Talent depositing 10 microliters of the diluted liposome suspension onto a carbon-coated copper grid.

3.2.2. Talent staining the grid with 2 percent uranyl acetate.

3.2.3. Talent placing the grid into the transmission electron microscope for imaging.

3.2.4. LAB MEDIA: 71788_shot_3.2.4_TEM.png

4. Light-Triggered Verteporfin Release

4.1. Prepare the VP-liposomes using the same lipid formulation, hydration, and extrusion procedures as demonstrated earlier [1]. Dispense 100 microliters of the liposome suspension in PBS into a dialysis device suspended in 12 milliliters of PBS, stirred at 80 rpm [2].

4.1.1. Shot of the prepared VP-liposomes.

4.1.2. Talent dispensing 100 microliters of the liposome suspension in PBS into a dialysis device suspended in 12 milliliters of phosphate-buffered saline.

4.2. Irradiate the samples with a 690-nanometer light-emitting diode light source at 0.15 milliwatts per square centimeter for 2, 4, and 6 minutes [1].

4.2.1. Talent irradiating the samples with a 690-nanometer light-emitting diode light source.

4.3. Sample the external dialysate at 0, 1, 3, 6, and 24 hours [1]. Quantify the released VP by fluorescence [2]. Obtain the total VP by lysing an equivalent aliquot with 0.1 percent Triton X-100 [3].

4.3.1. Talent collecting samples from the external dialysate.

4.3.2. Talent places the sample in the fluorescence spectrophotometer and note down the reading.

4.3.3. Talent lysing an equivalent aliquot with 0.1 percent Triton X-100.

4.4. Calculate the VP release using the presented equation [1].

4.4.1. TEXT ON A PLAIN BACKGROUND

$$R_{vp}(\%) = \frac{F_t - F_0}{F_{max} - F_0} \times 100$$

Where,

F_t = Fluorescence values at time t

F_0 = Baseline fluorescence

F_{max} = Fluorescence after Triton lysis

4.5. Measure the change in the hydrodynamic diameter by dynamic light scattering before and after irradiation to confirm membrane destabilization [1].

4.5.1. Talent placing the samples into the dynamic light scattering instrument.

5. In Vitro Transfection and Gene Knockout Assay

5.1. Seed the HEK293-GFP (*Heck-Two-Ninety-Three*) cells in 96-well plates at a density appropriate to achieve approximately 70 percent confluency at the time of treatment [1]. Culture the cells in a complete medium at 37 degrees Celsius and 5 percent carbon dioxide [2].

5.1.1. Talent seeding the HEK293-green fluorescent protein cells into a 96-well plate.

5.1.2. Talent placing the 96-well plate into the incubator.

5.2. Add the VP-liposomes encapsulating the anti-GFP single guide RNA and Cas9 RNP complex to the wells at the optimized concentration [1]. Incubate the cells for 4 hours to allow cellular uptake [2].

5.2.1. Talent adding the VP-liposomes to the wells of the 96-well plate.

5.2.2. Talent placing the 96-well plate into the incubator.

5.3. Irradiate the treated wells with a 690-nanometer LED source at 0.15 milliwatts per square centimeter for 20 minutes, alongside the light-only controls. The dark controls are not irradiated [1].

5.3.1. Talent irradiating the treated 96-well plate with a 690-nanometer light-emitting diode light source.

5.4. Assess the GFP knockout by fluorescence microscopy at 48 to 72 hours after the treatment [1].

5.4.1. SCREEN: 71788_shot_5.4.1_fluorescence.mov.

5.5. To measure the encapsulation efficiency, centrifuge a sample of the liposomes at

14,000 *g* for 1 hour at 4 degrees Celsius [1]. Filter the supernatant through a 20-nanometer membrane [2].

5.5.1. Talent placing the liposome sample into the centrifuge.

5.5.2. Talent filtering the collected supernatant through a 20-nanometer membrane.

5.6. Quantify the unencapsulated Cas9 protein in the filtrate by a bicinchoninic acid assay [1].

5.6.1. Talent adding the reagent to the filtrate.

5.7. Calculate the encapsulation efficiency using the equation [1].

5.7.1. TEXT ON A PLAIN BACKGROUND

$$EE (\%) = \left[\frac{C_{tot} - C_{sup}}{C_{tot}} \right] \times 100$$

Where,

C_{tot} = Total Cas9 added

C_{sup} = Unencapsulated Cas9 recovered in the supernatant

6. In Vivo Validation: Zebrafish Visual Reporter Assay

6.1. Prepare the injection Master Mix by combining the VP-liposomes with the single guide RNA at 0.01 micromolar and the Cas9 protein at 0.1 milligrams per milliliter [2].

6.1.1. Talent combining the VP-liposomes encapsulating the single guide ribonucleic acid and the Cas9 protein.

6.2. Using a calibrated microinjection system, inject 2 nanoliters of the Master Mix into the cell, rather than the yolk, of each one-cell-stage zebrafish embryo [1].

6.2.1. LAB MEDIA: 71788_shot_6.2.1_Embryo_Microinjection.png

6.3. At 2 hours post-fertilization, irradiate the injected embryos with a 690-nanometer LED light source at 0.15 milliwatts per square centimeter for a defined duration to activate cargo release [1].

6.3.1. Talent irradiating the injected zebrafish embryos with a 690 nanometer light-emitting diode light source.

6.4. Seventy-two hours after fertilization, anesthetize the embryos in 0.02% buffered Tricaine [1] and mount them in 1 percent low-melting-point agarose [2]. Using the smyhc1:eGFP (*S-M-Y-H-C-One-E-G-F-P*) reporter line, quantify slow-muscle fiber knockout by the loss of enhanced GFP fluorescence. Image the embryos using an inverted fluorescence microscope and a confocal microscope [3]. **NOTE: Added tricaine details as per author's email**

6.4.1. Talent anaesthetizing the embryos in Tricaine.

6.4.2. Talent mounting the embryos in 1 percent low-melting-point agarose.

6.4.3. **SCREEN:** 71788_shot_6.4.3_Inverted_Microscope.png.

6.5. Acquire focus-stacked images. Count the EGFP-negative slow-muscle fibers per embryo by analyzing approximately 16 embryos per group [1]. Prepare and characterize doxorubicin-loaded VP-gold nanoparticle-liposomes, then evaluate X-ray-triggered cargo release and cytotoxicity [2-TXT]. **NOTE: This VO is added to replace the extremely long text card given below, as authors want this section to be mentioned and discussed in the results**

6.5.1. **SCREEN:** 71788_shot_6.5.1 and 6.5.2 Fluorescent_Embryos.png.

6.5.2. *Reuse 2.1.1, 2.2.2 and 5.3.1* **TXT: See text protocol for VP/AuNP-liposome characterization and in vivo evaluation**

Video editor: Please use title card to show the following information

~~X-ray triggered VP-liposomes for chemotherapy drug delivery~~

- ~~• Prepare VP/AuNP-liposomes using DOTAP and DOPC, then incorporate VP and 3–5 nm gold nanoparticles.~~
- ~~• Extrude 11 times through a 200 nm membrane, then dialyze against PBS to establish an ammonium sulfate gradient.~~
- ~~• Load doxorubicin at a 1:10 drug-to-lipid weight ratio at 60 °C for 1 h, then remove unencapsulated drug by dialysis.~~
- ~~• Characterize the liposomes by DLS and determine doxorubicin encapsulation efficiency by fluorescence.~~
- ~~• Irradiate VP/AuNP-liposomes with 1, 2, or 4 Gy X rays and quantify cargo release by calcein fluorescence.~~
- ~~• Treat HCT116 cells with doxorubicin-loaded VP/AuNP-liposomes, irradiate with X rays, and include appropriate controls.~~
- ~~• Assess cell viability by MTS assay at 0, 2, 4, and 24 h after 4 Gy irradiation.~~

Results

7. Results

7.1. Light-activated embryos showed knockout of around 308.37 eGFP-positive slow-muscle fibers per embryo [1], compared with 53.15 fibers in the dark controls [2], increasing to about 326.12 fibers after 5 minutes of irradiation [3].

7.1.1. LAB MEDIA: Figure 1B. *Video editor: Highlight the Light(1min) and Light (2min) panels*

7.1.2. LAB MEDIA: Figure 1B. *Video editor: Highlight the No light panel*

7.1.3. LAB MEDIA: Figure 1B. *Video editor: Highlight the Light (5min) panel .*

7.2. In vitro, the light-activated VP-liposomes achieved GFP knockdown comparable to Lipofectamine 2000, while uniquely providing light-gated spatiotemporal control [1].

7.2.1. LAB MEDIA: Figure 1C. *Video editor: Highlight the bar labeled "light +" and +ve control bars in the left-hand plot.*

7.3. VP with gold nanoparticle liposomes showed X-ray dose-dependent calcein release at the doses of 1 to 4 gray [2].

7.3.1. LAB MEDIA: Figure 2B. *Video editor: Highlight the blue curve data points.*

7.4. Mice treated with doxorubicin-loaded VP with gold nanoparticle-liposomes followed by 4 gray X-ray irradiation showed significant tumor-growth suppression [1] compared with the Liposome-doxorubicin alone [2], the X-ray alone [3], and PBS [4].

7.4.1. LAB MEDIA: Figure 2C. *Video editor: Highlight the curve labeled "X-ray triggered LipoDox".*

7.4.2. LAB MEDIA: Figure 2C. *Video editor: Highlight the curve labeled "LipoDox".*

7.4.3. LAB MEDIA: Figure 2C. *Video editor: Highlight the curve labeled "X-ray".*

7.4.4. LAB MEDIA: Figure 2C. *Video editor: Highlight the curve labeled "PBS".*

7.5. Histological analysis revealed larger necrotic areas in the combined-treatment group [1], compared to the other control groups [2].

7.5.1. LAB MEDIA: Figure 2D. *Video editor: Highlight the arrows in the image and the box labeled "X-ray triggered LipoDox" in the right-hand plot.*

7.5.2. LAB MEDIA: Figure 2D. *Video editor: Highlight the boxes labeled PBS, LipoDox, X-ray*

1. CRISPR
Pronunciation link: <https://www.merriam-webster.com/dictionary/CRISPR>
IPA: /'krɪspər/
Phonetic Spelling: kris·per
2. Nuclease
Pronunciation link: <https://www.merriam-webster.com/dictionary/nuclease>
IPA: /'nu:kliˌeɪs/
Phonetic Spelling: noo·klee·ays
3. Ribonucleoprotein
Pronunciation link: <https://www.merriam-webster.com/dictionary/ribonucleoprotein>
IPA: /ˌraɪboʊˌnu:kliəʊˈproʊtiːn/
Phonetic Spelling: rye·boh·noo·klee·oh·proh·teen
4. Liposome
Pronunciation link: <https://www.merriam-webster.com/dictionary/liposome>
IPA: /'lɪpəˌsoʊm/
Phonetic Spelling: lip·uh·sohm
5. Multilamellar
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IPA: /ˌmʌltɪˌlæˈmɛlər/
Phonetic Spelling: mul·tee·lam·el·er
6. Polycarbonate
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Phonetic Spelling: pol·ee·kar·buh·nuht
7. Unilamellar
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Phonetic Spelling: yoo·nee·lam·el·er
8. Verteporfin
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IPA: /ˌvɜːrtəˈpɔːrfɪn/
Phonetic Spelling: ver·tuh·por·fin
9. Physicochemical
Pronunciation link: <https://www.merriam-webster.com/dictionary/physicochemical>
IPA: /ˌfɪzɪkoʊˈkɛmɪkəl/
Phonetic Spelling: fiz·ih·koh·kem·ih·kuhl
10. Hydrodynamic
Pronunciation link: <https://www.merriam-webster.com/dictionary/hydrodynamic>
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Phonetic Spelling: hy·droh·dy·nam·ik

11. Polydispersity
Pronunciation link: No confirmed link found
IPA: /ˌpɑːlɪdiˈspɜːrsəti/
Phonetic Spelling: pol·ee·dih·spur·suh·tee
12. Zeta
Pronunciation link: <https://www.merriam-webster.com/dictionary/zeta>
IPA: /ˈzɛtə/
Phonetic Spelling: zay·tuh
13. Microscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/microscopy>
IPA: /maɪˈkrɑːskəpi/
Phonetic Spelling: my·kros·kuh·pee
14. Uranyl
Pronunciation link: <https://www.merriam-webster.com/dictionary/uranyl>
IPA: /ˈjʊrənəl/
Phonetic Spelling: yur·uh·nuhl
15. Acetate
Pronunciation link: <https://www.merriam-webster.com/dictionary/acetate>
IPA: /ˈæsəˌteɪt/
Phonetic Spelling: ass·uh·tayt
16. Dialysis
Pronunciation link: <https://www.merriam-webster.com/dictionary/dialysis>
IPA: /daɪˈæləsis/
Phonetic Spelling: dy·al·uh·sis
17. Dialysate
Pronunciation link: <https://www.merriam-webster.com/dictionary/dialysate>
IPA: /daɪˈæliˌzeɪt/
Phonetic Spelling: dy·al·ih·zayt
18. Aliquot
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>
IPA: /ˈæliˌkwɑːt/
Phonetic Spelling: al·ih·kwot
19. Triton
Pronunciation link: <https://www.merriam-webster.com/dictionary/triton>
IPA: /ˈtraɪtən/
Phonetic Spelling: try·tuhn
20. Confluency
Pronunciation link: No confirmed link found
IPA: /ˈkɑːnfluənsi/
Phonetic Spelling: kon·floo·uhn·see
21. Transfection
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IPA: /trænsˈfɛkʃən/
Phonetic Spelling: trans·fek·shuhn

22. Encapsulation
Pronunciation link: <https://www.merriam-webster.com/dictionary/encapsulation>
IPA: /ɪnˌkæpsəˈleɪʃən/
Phonetic Spelling: en·kap·syuh·lay·shuhn
23. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
24. Supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /ˌsuːpərˈnɛtənt/
Phonetic Spelling: soo·per·nay·tuhnt
25. Bicinchoninic
Pronunciation link: No confirmed link found
IPA: /ˌbaɪsɪŋkoʊˈnɪnɪk/
Phonetic Spelling: by·sing·koh·nin·ik
26. Filtrate
Pronunciation link: <https://www.merriam-webster.com/dictionary/filtrate>
IPA: /ˈfɪlˌtreɪt/
Phonetic Spelling: fil·trayt
27. Microinject
Pronunciation link: No confirmed link found
IPA: /ˌmaɪkroʊmˈdʒɛkt/
Phonetic Spelling: my·kroh·in·jekt
28. Microinjection
Pronunciation link: No confirmed link found
IPA: /ˌmaɪkroʊmˈdʒɛkʃən/
Phonetic Spelling: my·kroh·in·jek·shuhn
29. Zebrafish
Pronunciation link: <https://www.merriam-webster.com/dictionary/zebrafish>
IPA: /ˈziːbrəˌfɪʃ/
Phonetic Spelling: zee·bruh·fish
30. Fertilization
Pronunciation link: <https://www.merriam-webster.com/dictionary/fertilization>
IPA: /ˌfɜːrtələˈzeɪʃən/
Phonetic Spelling: fur·tuh·luh·zay·shuhn
31. Anesthetize
Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthetize>
IPA: /əˈnɛsθəˌtaɪz/
Phonetic Spelling: uh·nes·thuh·tyze
32. Tricaine
Pronunciation link: No confirmed link found
IPA: /ˈtraɪkeɪn/
Phonetic Spelling: try·kayn

33. Agarose
 Pronunciation link: <https://www.merriam-webster.com/dictionary/agarose>
 IPA: /'ægəˌroʊs/
 Phonetic Spelling: ag·uh·rohs
34. Confocal
 Pronunciation link: <https://www.merriam-webster.com/dictionary/confocal>
 IPA: /kɑːn'foʊkəl/
 Phonetic Spelling: kon·foh·kuhl
35. Doxorubicin
 Pronunciation link: <https://www.merriam-webster.com/dictionary/doxorubicin>
 IPA: /ˌdɒːksə'ruːbəsin/
 Phonetic Spelling: dok·suh·roo·buh·sin
36. Nanoparticle
 Pronunciation link: <https://www.merriam-webster.com/dictionary/nanoparticle>
 IPA: /'nænoʊˌpɑːrtɪkəl/
 Phonetic Spelling: nan·oh·par·tih·kuhl
37. Cytotoxicity
 Pronunciation link: <https://www.merriam-webster.com/dictionary/cytotoxicity>
 IPA: /ˌsaɪtəʊtɪk'ɪsɪtɪ/
 Phonetic Spelling: sy·toh·tok·sis·uh·tee
38. Ammonium
 Pronunciation link: <https://www.merriam-webster.com/dictionary/ammonium>
 IPA: /ə'moʊniəm/
 Phonetic Spelling: uh·moh·nee·uhm
39. Calcein
 Pronunciation link: No confirmed link found
 IPA: /'kælsɪn/
 Phonetic Spelling: kal·see·in
40. Lipofectamine
 Pronunciation link: No confirmed link found
 IPA: /ˌlɪpoo'fektəmiːn/
 Phonetic Spelling: lip·oh·fek·tuh·meen
41. Spatiotemporal
 Pronunciation link: <https://www.merriam-webster.com/dictionary/spatiotemporal>
 IPA: /ˌspeɪʃioʊ'tempərəl/
 Phonetic Spelling: spay·shee·oh·tem·puh·ruhl
42. Histological
 Pronunciation link: <https://www.merriam-webster.com/dictionary/histological>
 IPA: /ˌhɪstə'lɑːdʒɪkəl/
 Phonetic Spelling: his·tuh·loj·ih·kuhl
43. Necrotic
 Pronunciation link: <https://www.merriam-webster.com/dictionary/necrotic>
 IPA: /nə'kraːtɪk/
 Phonetic Spelling: nuh·krot·ik

