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Title: Parallel *In Vivo* Screening of Gene Knockout and Activation in the Mouse Mammary Gland

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**

If **Yes**, can you record movies/images using your own microscope camera?

No

If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit.

If your microscope does not have a camera port, the scope kit will be attached to one of the eyepieces and **you will have to perform the procedure using one eye.**

We have several different microscope options. We have access to a teaching microscope so the unused eyepieces can be used for the scope kit, and might have a camera port (I'm not sure). I can't find the model number on the teaching microscope, but the eyepieces look to be the same as our Leica MS5.

If a dissection or stereo microscope is required for your protocol, please list all shots from the script that will be visualized using the microscope (shots are indicated with the 3-digit numbers, like 2.1.1, 2.1.2, etc.).

Scope shots: 2.8.1, 2.10.1, 2.10.2, 2.10.3 , 2.11.1, 2.11.2 , 2.12.1

2. Software: Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. Filming location: Will the filming need to take place in multiple locations? **Yes**

If **Yes**, how far apart are the locations? Across the street

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. **No**

Current Protocol Length

Number of Steps: 25

Number of Shots: 42

Interviews

Videographer: *Obtain headshots for all authors available at the filming location.*

INTRODUCTION:

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

- 1.1. **Ellen Langille:** Our research aims to identify cancer-causing genes through high-throughput screening while maintaining an intact tissue microenvironment in the mouse mammary gland.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~Describe the research contexts or systems in which this protocol can be applied.~~

- 1.2. **Ellen Langille:** This protocol enables functional interrogation of gene libraries in mammary epithelium using genetically engineered mouse models *in vivo*.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

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

- 1.3. **Ellen Langille:** The greatest challenge is achieving a high viral titer to maximize single-infected cells and obtain the highest coverage with fewer animals.
 - 1.3.1. 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?~~

- 1.4. **Ellen Langille:** Following this procedure, mice can be further analyzed using flow cytometry, spatial transcriptomics, or single-cell RNA sequencing technologies.
 - 1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

- 1.5. **Ellen Langille:** Future studies can adapt this method to other organs or incorporate Cas12 arrays and base editing technologies.

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

Videographer: Obtain headshots for all authors available at the filming location.

Ethics Title Card

This research has been approved by The Canadian Council on Animal Care and under protocols approved by The Centre for Phenogenomics Animal Care Committee at the Lunenfeld-Tanenbaum Research Institute

Protocol

***Videographer:** Please always record an audio slate while filming each shot mentioned in the shotlist.*

2. Preparation of Mice and Intraductal Lentiviral Injection

Demonstrator: Ellen Langille

- 2.1. To begin, obtain the required surgical instruments and concentrated lentiviral samples [1]. Using a micropipette needle puller, pull glass microcapillary needles to generate fine-tipped capillaries suitable for intraductal injection of 9 to 10 microliter volumes [2]. With a pair of fine forceps, break off the very tip of the needle [3].
 - 2.1.1. WIDE: Talent sitting with surgical instruments and lentiviral sample on the workbench. **Videographer's NOTE: Take 1 and 2 wide shot, take 3 close up**
 - 2.1.2. Close-up of the micropipette needle puller being pulled and generating a fine-tipped glass capillary needle. **Videographer's NOTE: Shot was taken later after hood and scope shots**
 - 2.1.3. Close-up of fine forceps breaking off the very tip of a pulled glass microcapillary needle.
- 2.2. Dilute lentiviral preparations to a final concentration of 10 million plaque-forming units per milliliter in sterile PBS supplemented with 0.05 percent weight per volume Fast Green FCF (F-C-F) dye [1].
 - 2.2.1. Talent adding PBS to the tube containing the lentiviral preparation.
- 2.3. Anesthetize an adult female mouse 8 to 20 weeks of age under 4% isoflurane with 1 liter per minute oxygen [1-TXT]. Apply sterile ophthalmic lubricant to both eyes to prevent corneal drying during the procedure [2].
 - 2.3.1. Talent administering anesthesia to the mouse. **TXT: Mice strains: Rosa26-LSL-Cas9, H11-Cas9, or Rosa-LSL-tdTomato**
 - 2.3.2. Talent applying sterile ophthalmic lubricant to both eyes of the mouse.
- 2.4. Secure the mouse in a supine position on a covered heating pad set to low to help regulate body temperature [1]. Maintain anesthesia using 2% isoflurane with 1 liter per minute oxygen through a nosecone throughout the procedure [2].
 - 2.4.1. Talent securing the mouse in a supine position on the covered heating pad. **Videographer's NOTE: 2.4.1 and 2.4.2 are combined in one shot.**
 - 2.4.2. Talent positions the nosecone to maintain anesthesia.

- 2.5. Using a sterile cotton swab, apply depilatory cream around the third thoracic and fourth abdominal nipples in a circular area of approximately 0.5 centimeter radius for 10 to 15 seconds [1]. With sterile gauze and sterile water, immediately remove the cream to prevent skin irritation [2].
 - 2.5.1. Talent applying depilatory cream around the third thoracic and fourth abdominal nipples with a sterile cotton swab.
 - 2.5.2. Talent removing the cream with sterile gauze and sterile water.
- 2.6. Swab the skin surrounding each nipple using sterile gauze sequentially with 70% isopropyl alcohol and iodine [1].
 - 2.6.1. Talent swabbing the skin surrounding each nipple with sterile gauze.
- ~~2.7. Inside a biosafety cabinet, place the mouse under a dissecting microscope to clearly visualize the nipples [1].~~
 - ~~2.7.1. Talent placing the mouse under the dissecting microscope inside the biosafety cabinet.~~ Videographer's NOTE: Shot removed
- 2.8. After cleaning the forceps with 70 percent ethanol, gently remove any dead skin overlaying the nipple to expose the ductal opening [1].
 - 2.8.1. SCOPE: Shot of dead skin overlaying the nipple being removed using fine forceps.
- 2.9. Load 8 microliters of the diluted virus into a pulled glass microcapillary [1].
 - 2.9.1. Talent loading diluted virus into the pulled glass microcapillary.
- 2.10. With a pair of fine forceps, hold the nipple area [1]. Gently insert the needle tip into the nipple opening [2]. Slowly dispense the viral suspension into the mammary duct over 5 to 10 seconds [3]. Videographer's NOTE: Scope shots from 2.10.1 to 2.11.2 are combined in one shot.
 - 2.10.1. SCOPE: Talent holding the nipple area with fine forceps.
 - 2.10.2. SCOPE: Talent inserting the needle tip into the nipple opening.
 - 2.10.3. SCOPE: Talent dispensing the viral suspension into the mammary duct.
- 2.11. Using a dissecting microscope, monitor the spread of Fast Green dye during the injection [1]. Confirm successful intraductal delivery by observing dye dispersion throughout the ductal network beneath the skin [2].
 - 2.11.1. SCOPE: Show Fast Green dye spreading during injection.
 - 2.11.2. SCOPE: Show dye dispersing throughout the ductal network beneath the skin.
- 2.12. Identify failed injections by observing dye pooling within the mammary fat pad adjacent to the nipple [1].
 - 2.12.1. SCOPE: Show dye pooling in the mammary fat pad adjacent to the nipple.

- 2.13. Repeat the injection procedure for the third thoracic and fourth abdominal mammary glands on both sides, injecting a total of four glands per mouse [1].
 - 2.13.1. Talent injecting the third thoracic and fourth abdominal mammary glands.
- 2.14. Discontinue anesthesia [1] and transfer the mouse to a clean recovery cage [2].
 - 2.14.1. Talent discontinuing anesthesia.
 - 2.14.2. Talent transferring the mouse to a clean cage.
- 2.15. Once the mouse is fully ambulatory and normal behavior is observed, return it to the designated biosafety housing [1].
 - 2.15.1. Talent returning the recovered mouse to designated biosafety housing.
- 2.16. Transfer the mouse to a clean cage that can be returned to standard housing [1]. After 3 days of injection, place the cage in the designated disposal area [2]. NOTE: VO narration is also edited for inverted shots.
 - 2.16.2. Talent transferring the mouse to a clean cage.
 - 2.16.1. Talent placing the cage in the designated disposal area. NOTE: 2.16.2 was placed before 2.16.1

3. Tissue Dissociation and CRISPR Library Sequencing

- 3.1. Clean the space with DNA Erase [1]. After euthanizing the mice, harvest mammary glands or mammary tumors at the desired timepoints [2-TXT]. NOTE: VO is added for the moved shot.
 - 3.2.1. Talent wiping the space with DNA Erase. NOTE: This shot was requested to move here.
 - 3.1.1. Talent harvesting mammary glands or mammary tumors from the mouse. TXT: Euthanasia: CO₂ inhalation followed by cervical dislocation NOTE: 3.1.1 and 3.2.2 are combined in one shot.
- 3.2. ~~Clean the space with DNA Erase [1].~~ After ensuring the equipment is free of plasmids containing single guide RNA or sgRNA (*S-G-R-N-A*) or dual guide RNA cassettes or dgRNA (*D-G-R-N-A*), digest each mammary gland in 2 milliliters of complete Epicult-B (*EH-pih-cult-B*) Medium supplemented with 0.2 milliliters of gentle collagenase hyaluronidase in a 15 milliliter conical tube [2]. Place the tube at 37 degrees Celsius overnight [3].
 - 3.2.1. ~~Talent wiping the space with DNA Erase.~~ NOTE: Move this before 3.1.1
 - 3.2.2. Talent adding mammary gland tissue to a 15 milliliter conical tube containing complete Epicult-B Medium Mouse.
 - 3.2.3. Talent placing the conical tube in the incubator.

- 3.3. Pellet dissociated cells at 450 g for 5 minutes at room temperature [1].
 - 3.3.1. Talent placing the tube into the centrifuge.
- 3.4. Using a bovine serum albumin-coated pipette tip, carefully transfer the upper fatty layer to a new 15 milliliter conical tube [1]. Discard the remaining supernatant [2].
 - 3.4.1. Talent transferring the upper fatty layer into a new 15 milliliter conical tube with a bovine serum albumin-coated pipette tip.
 - 3.4.2. Talent discarding the remaining supernatant.
- 3.5. Wash both the fatty fraction and the cell pellet once with 5 milliliters PBS [1]. Centrifuge at 450 g for 5 minutes at room temperature [2]. Discard the supernatant [3].
 - 3.5.1. Talent adding PBS onto the fatty fraction and cell pellet.
 - 3.5.2. Talent placing the tubes into the centrifuge.
 - 3.5.3. Talent discarding the supernatant from the tubes.
- 3.6. Combine the washed fatty fraction and the cell pellet from the same mammary gland into a single 15 milliliter conical tube containing 5 milliliters of PBS [1].
 - 3.6.1. Talent adding washed fatty fraction and cell pellet into a single conical tube.
- 3.7. Pellet the combined material at 450 g for 5 minutes [1]. Discard the supernatant [2].
 - 3.7.1. *Resuse 3.5.2.*
 - 3.7.2. Talent discarding the supernatant from the tube.
- 3.8. Using sterile scissors or a scalpel, mince the mammary tumor tissue into small chunks [1]. Transfer the minced tissue directly to a 2 milliliter microfuge tube [2].
 - 3.8.1. Talent mincing mammary tumor tissue into small chunks using sterile scissors or a scalpel.
 - 3.8.2. Talent transferring the minced tissue into a 2 milliliter microfuge tube.
- 3.9. Isolate genomic DNA from the harvested cells using a commercial genomic DNA extraction kit [1]. After isolating the genomic DNA (*D-N-A*), perform next-generation sequencing of CRISPR (*CRIS-per*) libraries and analyze the data [2].
 - 3.9.1. Talent sitting at the workbench, pipetting into a tube with a commercial genomic DNA extraction kit next to it. Videographer's NOTE: Take 1 wide shot, take 2 close up
 - 3.9.2. Show submission of CRISPR libraries for next-generation sequencing on the computer interface.

Results

4. Results

- 4.1. PCR amplification of guide RNA or gRNA (*G-R-N-A*) libraries yielded discrete bands [1], indicating uniform amplification from pooled oligonucleotide chip arrays [2].
 - 4.1.1. LAB MEDIA: Figure 2A. *Video editor: Emphasize the 3 bands visible in the column (+)*
 - 4.1.2. LAB MEDIA: Figure 2A.
- 4.2. Restriction digestion of the 2 lentiviral vector backbones resulted in clear separation of the linearized backbone from the stuffer fragment [1].
 - 4.2.1. LAB MEDIA: Figure 2B,C. *Video editor: In Figure 2B, emphasize the 2 bands labeled 8.6 kb backbone and 2 bands labeled 2.5 kb stuffer. In Figure 2C, emphasize the 2 bands labeled 7.5 kb backbone and 2 bands labeled 1.8 kb stuffer.*
- 4.3. Successful library cloning showed dense and evenly distributed colonies [1], while negative control plates contained few colonies [2].
 - 4.3.1. LAB MEDIA: Figure 3. *Video editor: Emphasize the plates labeled Backbone + Insert*
 - 4.3.2. LAB MEDIA: Figure 3. *Video editor: Emphasize the plate labeled Negative Control*
- 4.4. Efficient lentiviral preparations showed a dose-dependent increase in tdTomato (*T-D-Tomato*) expression [1].
 - 4.4.1. LAB MEDIA: Figure 4A,C. *Video editor: Emphasize the line along with the 4 dots on it.*
- 4.5. Low-titer and high-titer viral preparations produced distinct tdTomato expression patterns across increasing virus doses [1].
 - 4.5.1. LAB MEDIA: Figure 4B,D. *Video editor: Emphasize all 4 stacked plots.*
- 4.6. Proper placement of the glass microcapillary enabled access to the mammary duct [1].
 - 4.6.1. LAB MEDIA: Figure 5A. *Video editor: Circle and highlight the glass needle and the small opening.*
- 4.7. Successful injections showed uniform spreading of Fast Green dye throughout the ductal tree [1], whereas failed injections showed dye pooling beneath the nipple [2].

- 4.7.1. LAB MEDIA: Figure 5B. *Video editor: Emphasize the blue coloured second image.*
- 4.7.2. LAB MEDIA: Figure 5C. *Video editor: Highlight the concentrated pool of green dye.*
- 4.8. Immunohistochemical staining confirmed epithelial cell infection [1], and flow cytometry quantified luminal and basal epithelial cell infections. [2].
 - 4.8.1. LAB MEDIA: Figure 5D. *Video editor: Highlight the brown stained cells visible in the image labeled Lv-RFP.*
 - 4.8.2. LAB MEDIA: Figure 5E. *Video editor: Emphasize the boxed area in all plots, along with the percentages displayed.*
- 4.9. PCR amplification of recovered genomic DNA yielded lentiviral-specific products [1].
 - 4.9.1. LAB MEDIA: Figure 6. *Video editor: Emphasize the dark DNA bands at the positions labeled “430 bp pXPR502” and “300 bp pLKO”*
- 4.10. Occasional non-specific amplification was observed in some CRISPR-A (CRIS-per-A) samples [1], although strong expected bands indicated successful library recovery [2].
 - 4.10.1. LAB MEDIA: Figure 6A. *Video editor: Emphasize the faint upper band above the main DNA band in the lane labeled A.*
 - 4.10.2. LAB MEDIA: Figure 6A,B. *Video editor: Emphasize the strong lower band at the positions labeled 430 bp pXPR502 and 300 bp pLKO*
- 4.11. Quality-controlled libraries showed even representation of guide RNAs, with only a few missing guides [1].
 - 4.11.1. LAB MEDIA: Figure 7B
- 4.12. Sequencing of individual clonal tumors often revealed clonal enrichment of a single gRNA [1], which could be classified as a screen hit [2].
 - 4.12.1. LAB MEDIA: Figure 7C. *Video editor: Emphasize the dotted line rising up at 1000 in the “Clonal Tumor” plot*
 - 4.12.2. LAB MEDIA: Figure 7C. *Video editor: Emphasize the signal marked “Hit” in the “Clonal Tumor” plot*

Pronunciation guides:

- 1. Microcapillary
Pronunciation link: <https://www.howtopronounce.com/microcapillary>
IPA: /ˌmaɪkroʊˈkæpəˌlɛri/
Phonetic Spelling: my-kroh-kap-uh-lair-ee
- 2. Intraductal
Pronunciation link: <https://www.howtopronounce.com/intraductal>

IPA: /ˌɪntrəˈdʌktəl/

Phonetic Spelling: in·truh·duhk·tuhl

3. Lentiviral

Pronunciation link: <https://www.howtopronounce.com/lentiviral>

IPA: /ˌlɛntiˈvaɪrəl/

Phonetic Spelling: len·tee·vy·ruhl

4. Plaque-forming

Pronunciation link: <https://www.merriam-webster.com/dictionary/plaque>

IPA: /plæk ˈfɔːrmiŋ/

Phonetic Spelling: plak·for·ming

5. Ophthalmic

Pronunciation link: <https://www.merriam-webster.com/dictionary/ophthalmic>

IPA: /ɒfˈθælmɪk/

Phonetic Spelling: off·thal·mik

6. Corneal

Pronunciation link: <https://www.merriam-webster.com/dictionary/corneal>

IPA: /ˈkɔːrniəl/

Phonetic Spelling: kor·nee·uhl

7. Isoflurane

Pronunciation link: <https://www.merriam-webster.com/medical/isoflurane>

IPA: /ˌaɪsoʊˈflʊreɪn/

Phonetic Spelling: eye·soh·floor·ayn

8. Depilatory

Pronunciation link: <https://www.merriam-webster.com/dictionary/depilatory>

IPA: /dɪˈpɪləˌtɔːri/

Phonetic Spelling: dih·pih·luh·tor·ee

9. Thoracic

Pronunciation link: <https://www.merriam-webster.com/dictionary/thoracic>

IPA: /θəˈræsɪk/

Phonetic Spelling: thuh·ra·sik

10. Isopropyl

Pronunciation link: <https://www.merriam-webster.com/dictionary/isopropyl>

IPA: /ˌaɪsəˈproʊpəl/

Phonetic Spelling: eye·suh·proh·puhl

11. Anesthesia

Pronunciation link: <https://www.merriam-webster.com/dictionary/anesthesia>

IPA: /ˌænəsˈθiːzə/

Phonetic Spelling: an·uhs·thee·zhuh

12. Ambulatory

Pronunciation link: <https://www.merriam-webster.com/dictionary/ambulatory>

IPA: /ˈæmbjələˌtɔːri/

Phonetic Spelling: am·byuh·luh·tor·ee

13. Euthanizing
Pronunciation link: <https://www.merriam-webster.com/dictionary/euthanize>
IPA: /'ju:θəˌnaɪz/
Phonetic Spelling: yoo·thuh·nyz
14. Plasmids
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /'plæzmɪd/
Phonetic Spelling: plaz·mid
15. sgRNA
Pronunciation link: <https://www.howtopronounce.com/sgrna>
IPA: /ˌɛs.dʒiː.ɑːr.ɛn'eɪ/
Phonetic Spelling: ess·gee·ar·en·ay
16. dgRNA
Pronunciation link: <https://www.howtopronounce.com/dgrna>
IPA: /ˌdiː.dʒiː.ɑːr.ɛn'eɪ/
Phonetic Spelling: dee·gee·ar·en·ay
17. Collagenase
Pronunciation link: <https://www.merriam-webster.com/medical/collagenase>
IPA: /kəˈlædʒəˌneɪs/
Phonetic Spelling: kuh·la·juh·nays
18. Hyaluronidase
Pronunciation link: <https://www.merriam-webster.com/medical/hyaluronidase>
IPA: /haɪəˌlʊrəˈnaɪˌdeɪs/
Phonetic Spelling: hy·uh·lur·uh·nye·days
19. Epicult-B
Pronunciation link: No confirmed link found
IPA: /'epɪˌkʌlt biː/
Phonetic Spelling: eh·pih·cult·bee
20. Scalpel
Pronunciation link: <https://www.merriam-webster.com/dictionary/scalpel>
IPA: /'skælpəl/
Phonetic Spelling: skal·puhl
21. CRISPR
Pronunciation link: <https://www.merriam-webster.com/dictionary/CRISPR>
IPA: /'krɪspər/
Phonetic Spelling: kris·pur
22. Oligonucleotide
Pronunciation link: <https://www.merriam-webster.com/medical/oligonucleotide>
IPA: /ˌɑːlɪɡoʊˈnuːkliəˌtaɪd/
Phonetic Spelling: ol·ih·goh·noo·klee·uh·tyde
23. tdTomato
Pronunciation link: <https://www.howtopronounce.com/tdtomato>

IPA: /ˌtiː.diː.təˈmɛtʊ/

Phonetic Spelling: tee-dee-tuh-may-toh

24. Immunohistochemical

Pronunciation link: <https://www.merriam-webster.com/medical/immunohistochemistry>

IPA: /ɪˌmjuːnʊˈhɪstəʊˈkɛmɪkəl/

Phonetic Spelling: ih-myoo-noh-his-toh-keh-mih-kuhl

25. Epithelial

Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>

IPA: /ˌɛpəˈθiːliəl/

Phonetic Spelling: eh-puh-thee-lee-uhl

26. Flow cytometry

Pronunciation link: <https://www.merriam-webster.com/medical/flow%20cytometry>

IPA: /fləʊ saɪˈtəːmɛtri/

Phonetic Spelling: floh sy-taa-muh-tree

27. Lentiviral-specific

Pronunciation link: No confirmed link found

IPA: /ˌlɛntiˈvaɪrəl spəˈsɪfɪk/

Phonetic Spelling: len-tee-vy-ruhl spuh-sih-fik