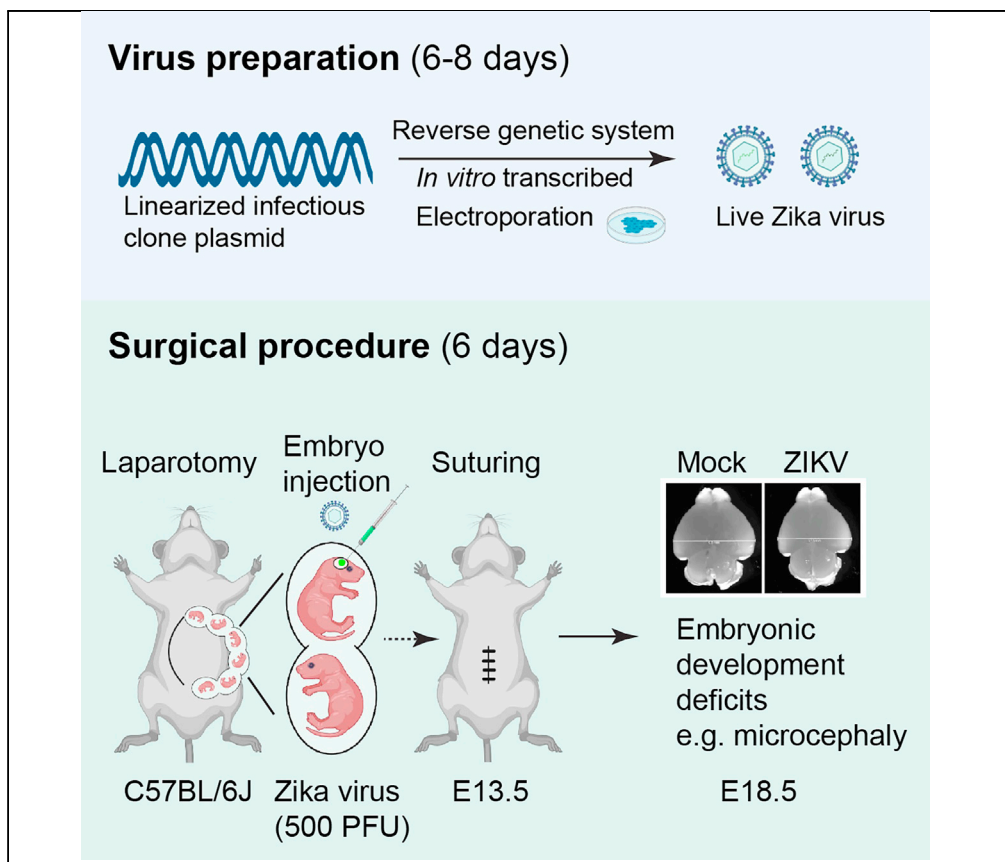


## Protocol

# Protocol for *in utero* injection to investigate Zika virus-related fetal microcephaly in mice



Zika virus (ZIKV) is linked to congenital defects including microcephaly. An infection model that can recapitulate most microcephaly-related phenotypes is crucial for understanding ZIKV pathogenesis. Here, we present a protocol to generate ZIKV from an infectious clone through a reverse genetic system and subsequently perform embryonic brain infection with the rescued ZIKV in pregnant mice. We optimized several aspects of the procedures including virus rescue and *in utero* injection. This protocol facilitates reproducible investigation of virus-induced cortical development defects.

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### Highlights

Workflow of Zika virus (ZIKV) preparation from an infectious clone

Protocol for *in utero* ZIKV injection

Details for brain sampling after ZIKV injection

Guidelines for ZIKV-related microcephaly phenotype analysis

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## Protocol

Protocol for *in utero* injection to investigate Zika virus-related fetal microcephaly in mice

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<https://doi.org/10.1016/j.xpro.2021.101057>

## SUMMARY

Zika virus (ZIKV) is linked to congenital defects including microcephaly. An infection model that can recapitulate most microcephaly-related phenotypes is crucial for understanding ZIKV pathogenesis. Here, we present a protocol to generate ZIKV from an infectious clone through a reverse genetic system and subsequently perform embryonic brain infection with the rescued ZIKV in pregnant mice. We optimized several aspects of the procedures including virus rescue and *in utero* injection. This protocol facilitates reproducible investigation of virus-induced cortical development defects.

For complete details on the use and execution of this protocol, please refer to Zeng et al. (2020)

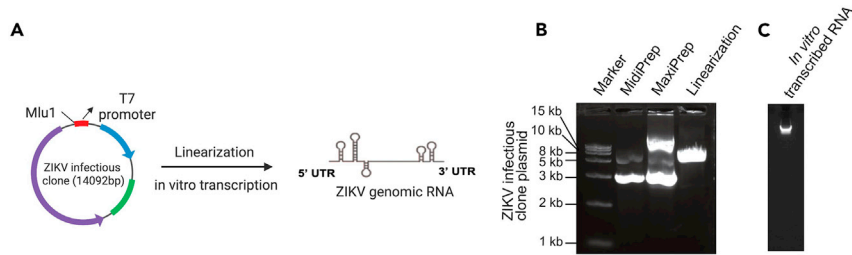
## BEFORE YOU BEGIN

## ZIKV preparation from an infectious clone

⌚ Timing: 8 days

1. Making ZIKV infectious clone plasmid
  - a. ZIKV infectious clone containing a T7 promoter (Zhao et al., 2018) (Figure 1A) is an ultralow-copy plasmid because it is cyclized through ZIKV genome sequence combined with Ampicillin resistant sequence. Maxiprep of the plasmid is necessary due to the large usage of the





**Figure 1. Preparation of *in vitro* transcribed ZIKV full-length RNA**

(A) The diagram for linearization by restriction enzyme Mlu1 and subsequent *in vitro* T7 transcription via a T7 promoter from a ZIKV infectious clone to finally produce ZIKV genomic RNA.

(B) Electrophoresis of MaxiPrep circular sample and linearized sample of ZIKV infectious clone plasmid.

(C) *In vitro* transcribed RNA from linearized ZIKV infectious plasmid for electrophoresis.

following steps. The concentration of infectious clone plasmid should be at least 200 ng/ $\mu$ L after MaxiPrep. If there is low purity and concentration of circular ZIKV infectious clone plasmid. [Troubleshooting 1](#)

**⚠ CRITICAL:** The purity of the plasmid has an important impact on the efficiency of *in vitro* transcription.

**⏸ Pause point:** Plasmids can be stored at  $-20^{\circ}\text{C}$  for months.

## 2. Linearization of ZIKV infectious clone plasmid

- Circular infectious plasmid DNA is needed to be linearized for *in vitro* transcription, using high fidelity Mlu1 enzyme in CutSmart Buffer at  $37^{\circ}\text{C}$  for 2 h following the manufacturer's protocol.
- The linearized DNA is purified using the phenol/chloroform method, with equal volume of phenol:chloroform:IAA (25:24:1) for extraction.
- The quality of linearized plasmid DNA should be confirmed with electrophoresis by comparing to uncut plasmid ([Figure 1B](#)), and the purity of the linearized DNA should be checked with a NanoDrop Spectrophotometer. The A260/A280 value of an ideal linearized DNA sample is 1.8, and A260/A230 value should be more than 2.0–2.2.

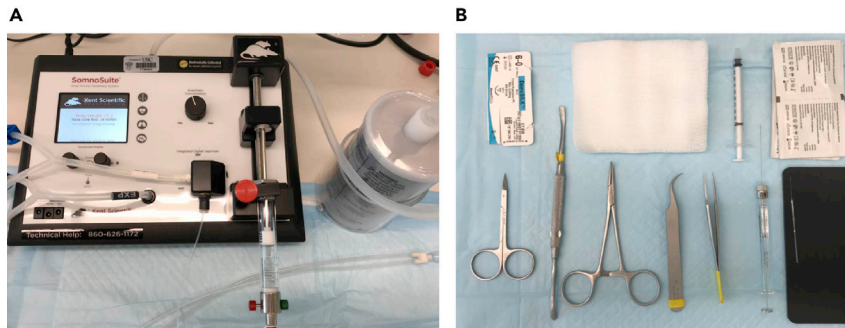
**⚠ CRITICAL:** DNA Gel Extraction Kit is not recommended for purifying the linearized plasmid because of the low isolation efficiency.

**⏸ Pause point:** Linearized plasmids can be stored at  $-20^{\circ}\text{C}$  for months.

## 3. *In vitro* transcription

- Linearized infectious plasmid DNA contains T7 promoter can be transcribed by *in vitro* using the mMESSAGE mMACHINE<sup>TM</sup> T7 Transcription kit Kit at  $37^{\circ}\text{C}$  for 2 h following the manufacturer's protocol (<https://www.thermofisher.com/order/catalog/product/AM1344?SID=srch-srp-AM1344>).
- Take  $\sim 2\mu\text{g}$  RNA products for electrophoresis to make sure its purity ([Figure 1C](#)), and the purity of the RNA products should be checked with a NanoDrop Spectrophotometer. The A260/A280 value of a good RNA sample is 2.0, and A260/A230 value should be more than 2.0–2.2.
- The remaining RNA products should be aliquoted into at 5  $\mu\text{g}$  per tube and stored at  $-80^{\circ}\text{C}$ , to avoid repeated freezing and thawing of synthetic RNA.

**Note:** Please carry out the following experience in RNase-free conditions. If you cannot see the turbidity after adding LiCl solution, please place it for 16 h at  $-20^{\circ}\text{C}$  to promote yield. We recommend dissolving the RNA production by 20  $\mu\text{L}$  RNase-free water firstly and then



**Figure 2. Preparation of the surgery related packs**

(A and B) Anesthesia machine (A) and composition of surgical packs (B). Surgical packs must be sterilized in an autoclave before surgery.

add appropriate RNase-free water according to the desired concentration. If low purity and yield of *in vitro* transcribed RNA is finally gotten. [Troubleshooting 2](#)

▮ **Pause point:** Transcribed RNA can be stored at  $-80^{\circ}\text{C}$  for months.

#### 4. ZIKV production from Vero cells

- $5\ \mu\text{g}$  *in vitro* transcribed RNA are mixed with  $\sim 5 \times 10^6$  Vero E6 cells in a 4 mm cuvette, and electroporated using the GenePulser apparatus with the settings of 450 V and 25  $\mu\text{F}$ , pulsing three times with 3 s intervals ([Shan et al., 2016](#)).
- The cells are seeded in the 10 cm cell culture dish with 10 mL DMEM medium supplemented with 2% FBS, and cultured in  $37^{\circ}\text{C}$ , 5%  $\text{CO}_2$  incubator.
- The virus supernatant should be harvested twice at day 5 and day 8 post electroporation, with brief centrifugation at  $2500 \times g$  for 10 min to remove cell debris, and stored in aliquots at  $-80^{\circ}\text{C}$ .
- The virus titer in the supernatant harvested at day 6 and day 8 post electroporation should be analyzed with plaque assay. The ideal virus titer achieved is  $10^7$ – $10^8$  PFU/mL.

**Note:** multiple electroporation reactions and subsequent cell cultures can be performed to increase the viral production. Additional cell samples can be prepared for harvesting cell lysates at 12, 24, 48, 72, 96 and 120 h post electroporation, to test viral replication over time by growth curve based qRT-PCR and viral load by western blot of NS5 or E protein.

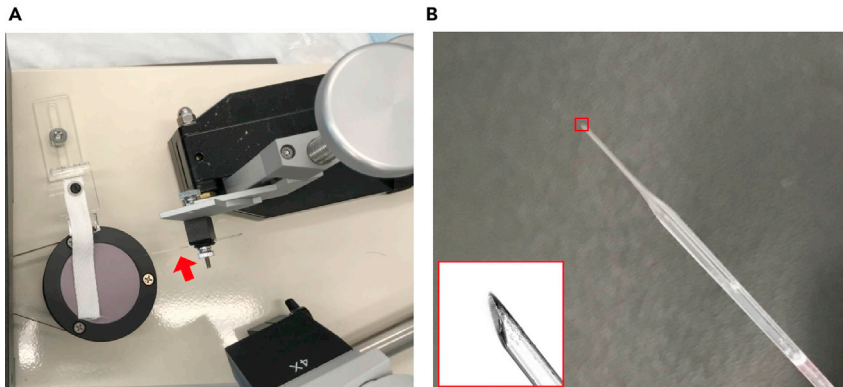
⚠ **CRITICAL:** These steps must be carried out in the hood of the corresponding biosafety level (BSL) laboratory and RNase-free environment. For instance, ZIKV-related experiments need a BSL-2 lab.

### Surgical instruments

⌚ **Timing:** 15 min

#### 5. Prepare surgical packs

- Check the isoflurane to ensure enough amount for the duration of at least one pregnant mouse.
- Turn on the anesthesia machine for prewarming 15 min in advance.
- Connect the heating mat to power. Warm the sterile PBS (50 mL in a conical tube) in a heating block.
- Put autoclaved surgical tools and surgical drapes on the surgical platform ([Figure 2](#)).



**Figure 3. Preparation of microcapillaries for virus injection**

(A and B) Pipette Grinder (Cat# BV-10, Sutter Instrument) (A) and grinded glass capillary (B). Red arrow in A indicates the glass being grinded. Red box in B indicates the grinded tip magnified by the microscope (ECHO, REVOLVE).

**Note:** It is recommended to use one drape per mouse.

6. Prepare virus injection packs
  - a. Preparation of microinjection glass micropipette.
    - i. Pull the glass micropipette and cut at 1/4–1/3 length of its pulled tip.
    - ii. Sharpen the micropipette tip at a 45° angle using a micropipette grinder with a water drip until the tip opening reaches 50–70  $\mu\text{m}$  (Herrlinger et al., 2018) (Figure 3).

**Note:** It's better to confirm the intact needle tips under a microscope at 400 $\times$ .

**△ CRITICAL:** Confirmation that the prepared needle is not blocked at all via sterile PBS instead of any virus before the formal injection is recommended.

## Mouse mating

⌚ **Timing:** 2–3 weeks

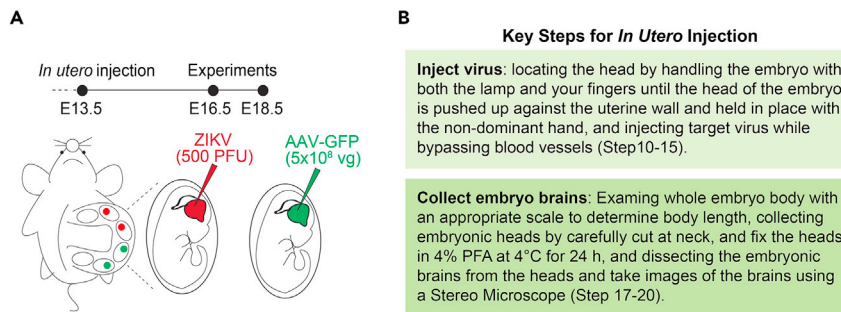
7. Mating C57BL/6J mice
  - a. Timed pregnancy in 10–12 weeks old C57BL/6J mice can be set up by checking females in the early morning for vaginal plugs to determine if mating has occurred. The day when the plugs are observed is referred to as E0.5.
  - b. The pregnant females are moved to separate cages, and we keep checking the mice every day before performing *in utero* injection at E13.5.

**Note:** We recommend to group-house females and synchronize their estrous cycles prior to mating. For detailed information for successful timed pregnancy, please check <https://www.jax.org/>

**△ CRITICAL:** Mating more female mice than the number needed for real experiments because unfertilized mating occurs in some cases. Alternatively, the timed pregnant mice are available commercially.

**KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
<b>Bacterial and virus strains</b>		
AAV-GFP	Zhen Zhao lab ( <a href="#">Zeng et al., 2020</a> )	n/a
<b>Chemicals, peptides, and recombinant proteins</b>		
phenol: chloroform: IAA (25:24:1)	Sigma-Aldrich	Cat # P2069
Sodium Acetate (3M), pH 5.5, RNase-free	Thermo Fisher Scientific	Cat # AM9740
UltraPure™ Distilled Water	Thermo Fisher Scientific	Cat # 10977023
4% formaldehyde, methanol-free	Thermo Fisher Scientific	Cat # FB002
Crystal Violet	Sigma-Aldrich	Cat # C0775
DMEM, powder, high glucose	Thermo Fisher Scientific	Cat # 12100046
Fetal Bovine Serum	Thermo Fisher Scientific	Cat # 10082147
Penicillin-streptomycin	Thermo Fisher Scientific	Cat # 15070063
Methyl cellulose	Sigma-Aldrich	Cat # M0512
Mlu1-HF	New England Biolabs	Cat # R3198S
10×CutSmart Buffer	New England Biolabs	Cat # B7204S
Tissue-Tek OCT compound	VWR	Cat # 25608-930
Sucrose	Sigma-Aldrich	Cat # S8501
Low melting point agarose	Thermo Fisher Scientific	Cat # 16520050
Triton X-100 solution	Thermo Fisher Scientific	Cat # 85111
Donkey serum	Jackson ImmunoResearch	Cat # 017-000-121
<b>Critical commercial assays</b>		
mMESSAGE mMACHINE™ T7 Transcription Kit	Thermo Fisher Scientific	Cat # AM1344
CompactPrep Plasmid Maxi Kit	QIAGEN	Cat # 12863
<b>Experimental models: cell lines</b>		
Vero E6 cell	ATCC	Cat # CCL-81
One Shot™ TOP10 Chemically Competent <i>E. coli</i>	Thermo Fisher Scientific	Cat # C404010
<b>Experimental models: organisms/strains</b>		
C57BL/6J mice	The Jackson Laboratory	stock 000664
<b>Recombinant DNA</b>		
ZIKV Brazil Infectious Clone pZL1	Gang Long Lab ( <a href="#">Zhao et al., 2018</a> )	n/a
<b>Other</b>		
Isoflurane	NDC	Cat # 13985-046-60
Buprenorphine	ZooPharm	Cat # Rx211750
Low-Flow anesthesia system	Kent Scientific Corporation	n/a
Dumont forceps-straight	Fine Science Tools	Cat # 11200-14
Narrow pattern forceps	Fine Science Tools	Cat # 11002-12
Fine scissors-straight	Fine Science Tools	Cat # 14060-10
Fine forceps-curved	Fine Science Tools	Cat # 11274-20
Combination spoon and spatula	Fine Science Tools	Cat # 10090-17
Hartman Hemostats-serrated	Fine Science Tools	Cat # 13002-10
Embedding mold	Polysciences, Inc	Cat # 18646A-1
DPBS	Thermo Fisher Scientific	Cat # 14190250
Mineral oil	Sigma-Aldrich	Cat # 330779
Micropipette Grinder	Sutter Instrument	Cat # BV-10
Micropipette Puller	Sutter Instrument	Cat # P-1000
Melting Point Capillary	KIMBLE	Cat # 34500-99
10 μL, Model 1701 LT SYR	HAMILTON	Cat # 80001
3× Surgical drapes 60 × 60 cm	Alcyon	Cat # 8337590
Heating mat 20.3 × 25.4 cm	Kent Scientific Corporation	Cat # RT-0502
Reagent Alcohol	VWR	Cat # 82018-050
Syringe 1 mL	Terumo	Cat # SS+01T1
Sterile gauze 5 × 7.5 cm	Coveto	Cat # 700099
Cordless Hair clipper type 1590	Wahl UK	n/a



**Figure 4. Schematic diagram of *in utero* injection**

(A) Schematic diagram of embryonic brain infection by ZIKV or AAV-GFP. Virus was micro-injected at lateral ventricle (LV) at E13.5, and experiments like body length were examined at E18.5. Sterile DMEM is used as sham injection control.

(B) Shown is key steps for *in utero* injection.

## MATERIALS AND EQUIPMENT

All equipment, reagents and consumables are listed in the table above. Alternative pipette pullers from other vendors could be used. Other types of drapes could be alternatively used such as disposable plastic drapes and cut the drapes to appropriate size if necessary.

## STEP-BY-STEP METHOD DETAILS

### *In utero* injection of virus

⌚ Timing: 1–2 h

Overall experimental workflow of *in utero* injection has been partially described (Herrlinger et al., 2018; Walantus et al., 2007; Yamada et al., 2008). *In utero* injection has been used to investigate ZIKV infection induced neurological deficits in fetus brain (Li et al., 2016; Shao et al., 2016; Yuan et al., 2017; Zhu et al., 2018). E13.5 embryos are subject to *in utero* injection with ZIKV or AAV-GFP virus (Figure 4).

**Note:** AAV-GFP here is primarily used to trace whether and where the delivered gene is expressed. Using this protocol, other viruses or materials like siRNA are also applicable.

1. Dilute ZIKV stock to  $5 \times 10^5$  PFU/mL or AAV-GFP stock to  $5 \times 10^{11}$  viral genomes per mL (vg/mL), aliquote the dilution as needed and keep the aliquot on ice.

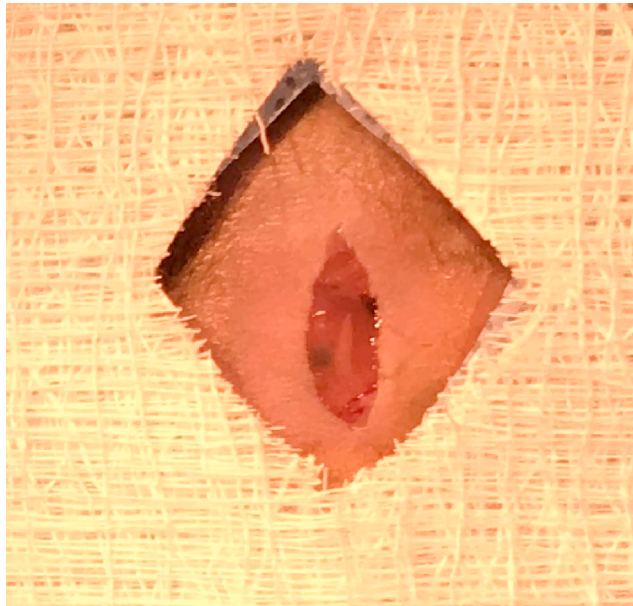
**Note:** Either DMEM or PBS is optional for dilution in the biological safety cabinet. The discarded solutions, tips, tubes, etc., should be soaked in 10% bleach.

2. Assemble the grinded pipette onto a Hamilton syringe, saturate the syringe with tubing with mineral oil and absorb about 8  $\mu$ L of virus aliquot into the pipette.

**Note:** Adjust the volume of absorbed virus aliquot according to the actual requirement.

**⚠ CRITICAL:** Loading the virus into the injection system and subsequent *in utero* injection should be performed in BSL-2 hood.

3. Anesthetize the dam with controllable isoflurane (Measured gas flow: 0.5 L/min; Anesthesia: 3% for induction and 2% for maintaining) by inhalation delivery while waste gas scavenging is



**Figure 5. Creating abdominal opening**

Trim the abdominal hair and sterilize the abdomen with iodophor and subsequently 70% alcohol. Create a 1–1.5 cm length opening via scissor to make the embryos exposed. Cover the mouse with both sterilized drape and gauze while the opening is exposed for injection.

present. Pinch and check the anesthetized mouse on the toe tip by forceps. Weigh the mouse and inject buprenorphine-SR (0.5–1.0 mg/kg) subcutaneously at the neck to induce analgesia.

**Note:** It's mandatory to document the usage history of buprenorphine-SR appropriately.

△ **CRITICAL:** The toe pinch forceps are no longer sterile and thus are not suitable for touching embryos later.

4. Lay the anesthetized mouse supine on an animal-friendly heating pad with appropriate size and add ophthalmic ointment to the eyes.

**Note:** Do not forget to use the ointment in order to protect the eyes from drying.

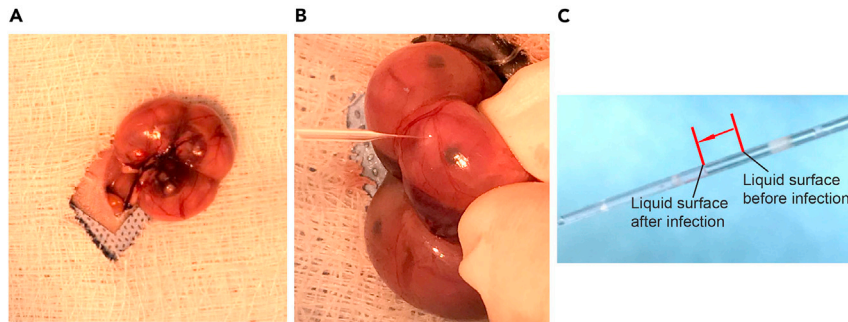
5. Trim the fur on the abdomen via veterinary hair clipper and disinfect with iodine and 70% alcohol at least three times. Drape the skin surrounding the surgical site with clean cloth to prevent contamination of the incision and the tools.

**Note:** Alternate the iodine and alcohol wipes and wipe in a circular motion away from the surgical site.

△ **CRITICAL:** The drape opening (1.5–2 cm width rhombus) must be larger than the surgical site than you plan. Carefully check and avoid any pollutant like hair or string in the drape opening.

6. Pinch the mother's skin a little away from her abdomen with sterile forceps and use sterilized scissors to cut the abdomen at the place of 1 cm away from vaginal opening and towards the head continue to cut into the skin and abdominal cavity for 1–1.5 cm along the medial sagittal line until the embryos are exposed (Figure 5).





**Figure 6. Virus microinjection**

(A) Take 3–4 embryos out from the abdomen one time and hydrate the embryos with sterile and warm PBS to avoid drying out.

(B) Left hand is used to stabilize the embryo and make an injection with right hand while the injection site must bypass blood vessel.

(C) Schematic diagram of 1  $\mu$ L volume of liquid injection by marked pipette, which helps judge whether the product has been really injected. Red arrow indicates movement direction of liquid in the pipette during injection.

- Cut a slit in the middle of a sterile gauze with appropriate size and put on top of the surgical opening. Hydrate the surgical opening with sterile and warm PBS. Pull the embryos out through the slit on the gauze.

**Note:** There are often 4–5 embryos in one uterine horn and thus only no more than 5 embryos can be pulled out at one time.

- Gently place a spatula under the head of the embryo. Lighten the embryos with a lamp to visualize the skull sutures, which help navigate the inject site later.

**△ CRITICAL:** Hydrate the surgical opening with sterile and warm PBS (37°C maintained in a heating block) during the surgery to prevent them from drying out.

- Locate the head by handling the embryo with both the lamp and your fingers until the head of the embryo is pushed up against the uterine wall and held in place with the non-dominant hand. If unideal injection position causes final low virus load. [Troubleshooting 3](#)

**Note:** Record the relative position of the embryos injected by different viruses, if possible, because embryos usually do not change their relative location during developing in utero. It is often suggested that the embryos at one uterine horn (usually 4–5 ones) as one group are injected with one virus.

**△ CRITICAL:** The embryo positioning should be practiced well because it is very important for successful surgery. Excessive finger pressure is likely to impair delicate embryonic membrane eventually causing embryo death later, while inadequate pressure could delay the injection process to make the embryo in danger.

- Employ the blood vessel running along the skull suture as a navigation. Inject ZIKV (1  $\mu$ L, equivalent to 500 PFU virus) or AAV-GFP (1  $\mu$ L, equivalent to  $5 \times 10^8$  vg) into the lateral ventricles of E13.5 embryo brains (Figure 6). Either sterile DMEM or PBS is used as a sham injection control accordingly.

**Note:** Marking each 1  $\mu$ L increment on the pipette outer surface using a marker pen is an option to help judge successful injection.

- △ **CRITICAL:** Carefully avoid blood vessels when choosing the injection site on embryonic brain to prevent hemorrhage and consequently embryo lethality.
  - △ **CRITICAL:** Operate the injection as fast as possible at no cost of successful rate to decrease the time of the whole surgery and thereby increase survival rate of both mother and embryo.
11. Put the injected embryos back into the pregnant dams. Hydrate the abdominal cavity with sterile PBS at last time. In a two-round interrupted suture, first suture the abdominal peritoneal muscle and then suture the external skin layer.
    - △ **CRITICAL:** Suturing is very critical for improving maintenance of the pregnancy because it is likely to happen that, due to skin inflammation, the mouse tends to scratch and chew the suture to cause wound rupture. In some serious cases, embryos are even exposed outside. Thus, the suture should be performed very carefully.
  12. Place the mouse in a cage partially heated by a heating pad or light to avoid body temperature reduction during anesthesia. Check frequently mothers in the cage until they recover from anesthesia.
  13. Put the cage back to the mouse room and develop the embryos after surgery for varied time according to the experimental plan. If Low survival rate of the pregnancy subjected to *in utero* injection happened at the endpoints. [Troubleshooting 4](#)

**Note:** Considering the mother after the surgery at the abdomen, it is difficult to move to drink water or stand to eat food, animal food and additional not-wetting water gel are better to be placed on cage ground.

### Collecting embryonic heads

⌚ **Timing:** 30 min

This major step accomplishes embryonic head collection at experimental endpoint.

14. Euthanize the mother with E16.5 or E18.5 embryos by CO<sub>2</sub>. Place the euthanized mother on the pad. Using forceps and scissors take the embryo out from the uterine horn. Put the embryos on another clean pad and take a picture of the whole embryo body with an appropriate scale to determine body length.
15. Collect embryonic heads by carefully cutting at the neck and fix the heads in 4% PFA at 4°C for 24 h.

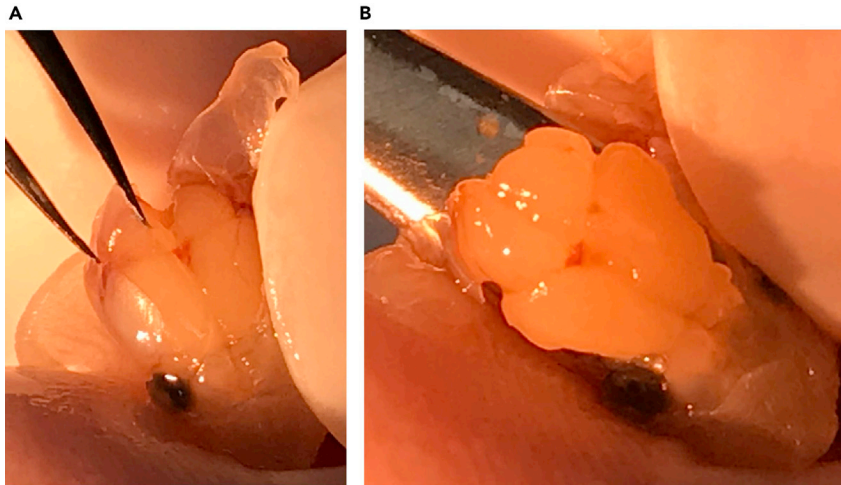
△ **CRITICAL:** It is recommended to perform the procedure in step 15 on a platform on top of ice.

### Examine embryonic brain width

⌚ **Timing:** 1–2 days

This major step accomplishes to examine microcephaly related phenotypes.

16. Wash the fixed heads with clean PBS for 5 min each time (at least 5 times). Dissect the embryonic brains from the heads ([Figure 7](#)) and take images of the brains using a Stereo Microscope (Leica, M165FC) or another available microscope to examine brain width.



**Figure 7. Collect embryonic brains**

(A) After 4% PFA fixation, remove soft skin and skull via sharp forceps while carefully avoiding to damage brain.  
(B) Take the brain out carefully via a flat spoon to ensure no damage of brain surface.

**Note:** Washing the heads with PBS as complete as possible because potential evaporated PFA is harmful.

△ **CRITICAL:** When dissecting the embryonic brains, the skin and skull should be gently removed to avoid damaging soft brain tissue and keep its completeness as much as possible. For this, dissection should be done under an appropriate stereoscope.

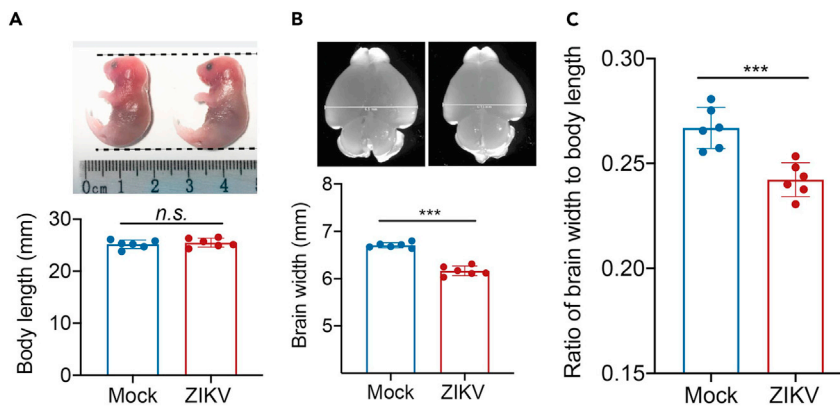
17. For cryo-sectioning, fixed brain tissues are cryoprotected in 30% sucrose in PBS for 16 h at 4°C and embedded in Tissue-Tek OCT compound. Cryostat sections are cut at 20 μm thickness. For vibratome sectioning, fixed brains are embedded in 5% low melt agarose and cut at 30 μm thickness.
18. Brain sections are permeabilized in PBS-T (PBS containing 0.2% Triton X-100) for 10 min, blocked with 5% normal donkey serum for 60 min, followed by observation with fluorescent microscope for GFP signal or incubation of scheduled primary antibodies.

**Note:** Coronal or sagittal sectioning is determined based on the histological perimeters.

△ **CRITICAL:** It's important that three comparable cryosections from each brain at 100 μm intervals should be immune-stained, from which appropriate quantification is performed.

## EXPECTED OUTCOMES

In our experience, about 80% in utero injections are successfully delivered regarding normal retention of pregnancy after appropriate and constant practice. No difference of body length of the E18.5 embryos is observed between mock and ZIKV infection (Figure 8A). But ZIKV infected embryos exhibited smaller brains, based on the measurements of brain width at E18.5 (Figure 8B). Ratio of brain width to body length is considered as an ideal parameter to evaluate microcephaly in mice. Consequently, the ratio in the ZIKV infected group is much lower than that in the mock group (Figure 8C), indicating ZIKV infection causes neurogenic deficits. To assess in utero injection models, AAV-GFP is used to trace injection. As a result, GFP is expressed well and is limited in brain parenchyma, as shown in sagittal (Figure 9A) or coronal (Figure 9B) section of E18.5 brain. In addition, ZIKV is also successfully injected at LV as shown by a strong signal of viral envelope protein distributed around the LV in E16.5 brain (Figure 9C). Thus, in utero injection is an ideal infectious model to investigate neurological deficits caused by viruses or other insults.



**Figure 8. Analysis of ZIKV related microcephaly phenotypes**

(A) Representative images of culture media- and ZIKV-infected fetal mice (upper panel) and according quantification (bottom panel) of body length in E18.5 fetal mice.

(B) Representative brain images of culture media- and ZIKV-infected fetal mice (upper panel) and according quantification (bottom panel) of brain width.

(C) Ratio of brain width in B to body length in A.  $n = 6$ . Mean  $\pm$  SD; \*\*\* $p < 0.001$  by Student's  $t$  test.

## LIMITATIONS

*In utero* injection in the mouse embryonic brain allows the manipulation of target gene expression or virus invasion during brain development and thus a powerful tool to investigate embryonic neurological deficits like microcephaly. As used in this study, accuracy, and consistency is partially based on constant practice. Other existing technologies could be considered to be used to enhance injection accuracy, such as high-frequency ultrasound imaging that permits the generation of images that can be used in real time to guide a microinjection needle into the embryonic region of interest (Piefelice and Gaiano, 2010). *In utero* injection is not suitable for studying mother-to-fetus transmission of a virus.

## TROUBLESHOOTING

### Problem 1

Low purity and concentration of circular ZIKV infectious clone plasmid purified by MaxiPrep DNA Extraction Kit. Related to step 1 in “before you begin”.

### Potential solution

We recommend eluting the DNA from the column by the elution buffer following the Kit protocol. Then precipitate DNA from the eluted sample via isopropanol and finally dissolve the DNA by fewer DNase/RNase-free water to reach a higher concentration.

### Problem 2

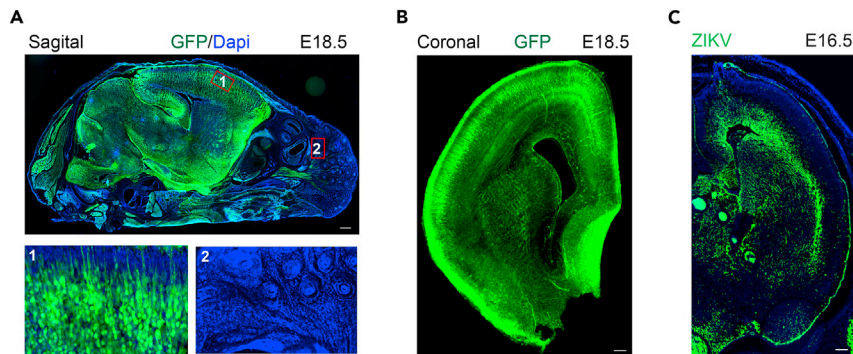
Low purity and yield of *in vitro* transcribed RNA. Related to step 3 in “before you begin”.

### Potential solution

Check the purity and concentration of circular and linearized ZIKV infectious clone plasmid.

Check the reagents NTP/CAP (2 $\times$ ), GTP (30 mM), and T7 Reaction Buffer (10 $\times$ ) for precipitation that will impact the transcription efficiency.

Do not discard the LiCl/transcription sample mixture if you just place it at  $-20^{\circ}\text{C}$  for only 30–60 min. Leave it 16 h at  $-20^{\circ}\text{C}$  and check it the next day.



**Figure 9. Detection of *in utero* injected viruses**

(A and B) Embryonic brains were infected at LV with AAV-GFP at E13.5 and the GFP expression in E18.5 embryonic brain at sagittal (A) or coronal (B) section.  
(C) Embryonic brains were infected at LV with ZIKV at E13.5 and the ZIKV envelop expression in E16.5 embryonic brain at coronal section. Scale bar: 0.5 mm.

Electrophoresis testing must be less than 5 min and in RNase-free condition to avoid RNA degradation.

### Problem 3

Unilateral injection position results in low virus load. Related to step 9 in “[step-by-step method details](#)”.

### Potential solution

This is another aspect to be more practice, however some tips are helpful to improve the injection.

AAV-GFP or other fluorescent protein is an ideal and convenient tool to indicate the consequence of the injection.

As described in steps 8–10 above, it is important to adjust the position of embryos by your fingers to a place where you are comfortable and convenient to perform injection.

Visible sagittal and coronal sutures on the surface of the embryonic brain are an ideal reference coordination system to consistently locate your injection site and direction toward the LV.

### Problem 4

Low survival rate of the pregnancy subjected to *in utero* injection. Related to step 13 in “[step-by-step method details](#)”.

### Potential solution

We recommend to constantly optimize whole process as below:

It should be very strict to sterilize all materials for the surgery, wear face mask, and changes the glove if it is touched by the contaminants during the surgery.

It’s better to have assistance from another person during the surgery to minimize the chance of your hand touching potential pollutants.

Perform the infection as fast as possible (~3 min each embryo is recommended) to minimize exposure time of the embryos.

It should be strict de-contaminate the surgical site in abdomen in combination with analgesia to minimize inflammation.

Do not use excessive finger pressure on the embryo to avoid damaging the yolk sac.

Make sure the infection site away from blood vessel on embryonic brain to avoid possible hemorrhage and consequently embryo lethality.

Make sure the sturdy suturing considering possible mouse scratch and chew the suture.

### RESOURCE AVAILABILITY

#### Lead contact

Further information and request for resources and reagents should be directed to and will be fulfilled by lead contact Jianxiong Zeng ([zengjianxiong@mail.kiz.ac.cn](mailto:zengjianxiong@mail.kiz.ac.cn)).

#### Materials availability

This study did not generate new unique reagents, cell, or mouse lines.

#### Data and code availability

This study did not generate a new dataset.

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### AUTHOR CONTRIBUTIONS

Q.L., Z.Z., and J.Z. conceived of the study. J.Z., S.D., and X.X. performed the experiments or wrote the protocol. G.L. and Y.C. provided ZIKV reverse genetic system. All authors commented on the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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