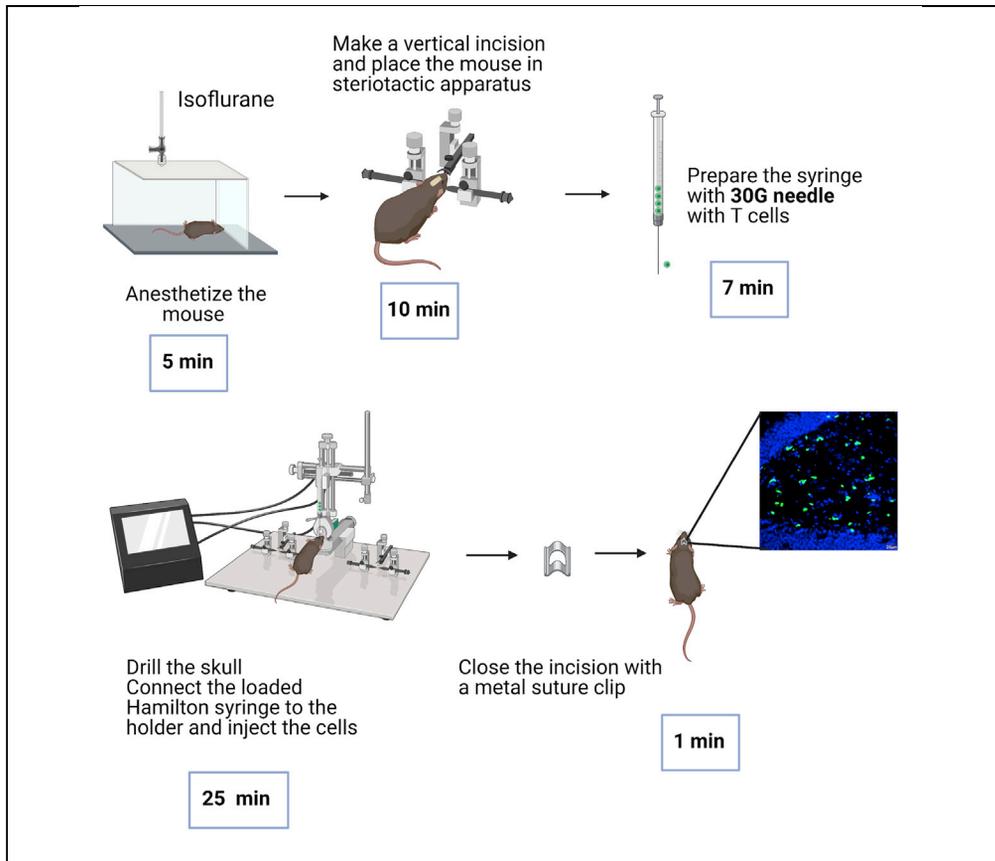


Protocol

An optimized intracerebroventricular injection of CD4⁺ T cells into mice



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Highlights

Bilateral intracerebroventricular injection of T cells using an optimized needle gauge

Reduced damage and increased survival of the injected cells

Enhanced migration of the injected cells within the brain parenchyma

The blood-brain barrier acts as a major barrier for the entrance of most therapeutics into the brain, impeding treatment for neurological disorders. Intracerebroventricular (ICV) injection of T cells is a useful tool for cell therapy of neurological disorders including neurodegenerative and neuropsychiatric diseases and brain tumors. Here, we present an optimized ICV injection of T cells with improved injection efficiency at pathological sites within the brain parenchyma. We describe details of the surgical procedure and verification of injection via immunohistochemistry.

Taylor et al., STAR Protocols 2, 100725
September 17, 2021 © 2021
The Authors.
<https://doi.org/10.1016/j.xpro.2021.100725>



Protocol

An optimized intracerebroventricular injection of CD4⁺ T cells into mice

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SUMMARY

The blood-brain barrier acts as a major barrier for the entrance of most therapeutics into the brain, impeding treatment for neurological disorders. Intracerebroventricular (ICV) injection of T cells is a useful tool for cell therapy of neurological disorders including neurodegenerative and neuropsychiatric diseases and brain tumors. Here, we present an optimized ICV injection of T cells with improved injection efficiency at pathological sites within the brain parenchyma. We describe details of the surgical procedure and verification of injection via immunohistochemistry.

For complete details on the use and execution of this protocol, please refer to Fisher et al. (2014); Strominger et al., (2018); Mittal et al. (2019); Eremenko et al. (2019).

BEFORE YOU BEGIN

⌚ Timing: 1–2 days

Intracerebroventricular (ICV) administration of cells in mouse models is an efficient tool for exploring the therapeutic efficacy of cells injected directly into the central nervous system (CNS) and thus bypassing the blood-brain barrier. ICV injection has been implemented for the administration of stem cells for various neurological conditions (Scruggs et al., 2013; Sironi et al., 2017). Notably, a recent phase 1 study safely injected adipose-derived stromal vascular fraction (ADSVF) into the human brain ventricular system of patients with neurodegenerative disorders. The authors reported a clinical improvement or stability of AD and amyotrophic lateral sclerosis (ALS) patients (Duma et al., 2019). We thus highlight the importance of this technique which presents an advantageous route of cell administration such as chimeric antigen receptor (CAR)-T cells targeted to brain malignancies (Bagley et al., 2018; Hunter and Jacobson, 2019; Majzner et al., 2019; Parker et al., 2020), or brain antigen-specific T cells targeted to amyloid plaques in mouse models of Alzheimer disease (Fisher et al., 2014; Strominger et al., 2018; Mittal et al., 2019; Eremenko et al., 2019). The protocol below describes the specific steps for injecting CD4⁺ T cells which were retrovirally transduced to express a green fluorescent protein (GFP). Although these cells were used to aid the better visualization of the injected cells, this protocol is compatible with all CD4⁺ T cells, which can be later stained with a suitable antibody to enable visualization.



Depending on your experimental needs, it is possible to inject unilaterally or bilaterally. For instance, unilateral ICV injections are suitable for cancer treatment studies ([Majzner et al., 2019](#); [Tanaka et al., 2020](#)). As we were interested in the interaction between the injected cells and neural cells, we preferred a bilateral injection. Hence, our protocol focuses on bilateral ICV injections.

1. Sterilize all the surgical tools.
2. Prepare mice for experiment (in this protocol we used 12 weeks C57BL/6JRcHsd male mice).

Note: Mice are housed under standard conditions. All procedures are performed under the approval of the Ben-Gurion University of the Negev Animal Care and Use Committee. All efforts are made to minimize suffering of the animals.

3. Setup the microinjection system.

Note: Ensure the infusion pump is set to run at a rate of 1 $\mu\text{L}/\text{min}$.

4. Ensure there is a sufficient amount of oxygen in the balloon.
5. Fill the VetFlo vaporizer with isoflurane.
6. Prepare the cells for injection. In microcentrifuge tubes, prepare 0.5×10^6 CD4⁺ GFP T cells in complete RPMI media. Prior to injection, wash cells with 1 mL PBS and suspend in 2.5 μL PBS.

Note: refer to ([Eremenko et al., 2021](#)) for the detailed protocol of isolating CD4⁺ T cells from mice and transduction with GFP-encoding retrovirus.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Isoflurane	Piramal Critical Care	NDC66794-017
RPMI Medium 1640	Gibco	21875
Fetal Bovine Serum	Gibco	10500-064
β -Mercaptoethanol (14.3 M)	Sigma-Aldrich	M3148
Sodium Pyruvate (100 mM)	Biological Industries	03-042-1B
MEM Non-Essential Amino Acids Solution (10 mM)	Biological Industries	01-340-1B
HEPES Buffer Solution (1M)	Biological Industries	03-025-1B
Penicillin-Streptomycin Solution (100 \times)	Biological Industries	03-031-1B
BupaqR Multidose 0.3 mg/mL Veterinary (Buprenorphine)	Richter Pharma	GTIN: (01) 900411402258
Ethanol 99.9% Tech	Romical	19-009101-78
4% Paraformaldehyde	Electron Microscopy Sciences	15714
Sucrose	Glentham Life Sciences	GC4473
O.C.T. Compound	Scigen	4586
Ethylene Glycol	ChemCruz	sc-257515A
Glycerol	Biolab	000712050100
Dulbecco's Phosphate Buffered Saline, without calcium and magnesium	Biological Industries	02-023-1A
Tween-20	Sigma-Aldrich	P1379
Triton X-100	Sigma-Aldrich	T9284
DAPI (4,6-diamidino-2-phenylindole)	BioLegend	422801
Anti-Fade Fluorescence Mounting Medium	Abcam	ab104135
Trypan Blue Solution	Biological Industries	03-102-1B

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
CD4 ⁺ GFP T Cells (isolated from C57BL/6JRccHsd mice and transduced with GFP-encoding retrovirus)	Refer to Eremenko et al., 2021 for the detailed protocol	N/A
Experimental models: Organisms/strains		
Mouse: C57BL/6JRccHsd	Envigo	2BL/6R04
Software and algorithms		
Allen Brain Atlas	https://portal.brain-map.org/	N/A
Other		
Quintessential Stereotaxic Injector with Digital Readout	Stoelting	53311
DC Temperature Controller	FHC	40-80-8D
VetFlo™	Kent Scientific Corporation	VetFlo-1205S
Induction Chamber	Kent Scientific Corporation	VetFlo-0720
Binocular Zoom Stereo Microscope	Motic	SMZ140
DC Temperature Controller	FHC	40-90-8D
Hamilton 25 µL Syringe	Hamilton	7636-01
Hamilton Gauge 26-gauge Point Style 3 Needle Length 2 inch	Hamilton	7768-02
Hamilton Gauge 30-gauge Point Style 3 Needle Length 2 inch	Hamilton	7762-03
Scalpel Handle	Bar Naor	BN10-104-04
Surgical Blades	Bar Naor	BN400-21-JH
Microdrill	RWD	68605
Reflex™ 7 mm Clip Applier	Kent Scientific	INS750343
Reflex™ Clips, 7 mm	Kent Scientific	INS750344
Cotton Tipped Applicators Non sterile	Bar Naor	BN72340631NS
Cryostat	Leica	CM3050S
Microscope Slides	Bar Naor Ltd.	BN9308C
Microscope Cover Glass	Bar Naor Ltd.	BN1052431STC
Microtome Blades	Leica	14035838925
12 Well Plate	Nunc	150628
6 Well Plate	Nunc	140675
48 Well Plate	Nunc	150687
15 mL Centrifuge Tube	Greiner	188261
Tissue-Tek Cryomold	Sakura	4557
Glass Pasteur Pipette	Glassco	GL 24672.380
FLUOVIEW FV1000 Laser-Scanning Confocal Microscope	Olympus	IX81-ZDC

Alternatives: In theory, all reagents and resources listed in the ‘key resources table’ can be substituted with equivalent items from other suppliers; however, it should be noted that the protocol has been calibrated to the reagents listed in the above table and alternatives have not been tested on the protocol performance.

MATERIALS AND EQUIPMENT

Complete RPMI medium		
Reagent	Final concentration	Amount for 500 mL
Fetal Bovine Serum	10% (v/v)	50 mL
Penicillin-Streptomycin Solution (100×)	1 mg/mL Streptomycin, 100 U/mL Penicillin	5 mL
HEPES Buffer Solution (1M)	10 mM	5 mL
MEM Non-Essential Amino Acids Solution (10 mM)	0.1 mM	5 mL
Sodium Pyruvate (100 mM)	1 mM	5 mL
β-mercaptoethanol (14.3 M)	50 µM	1.75 µL
RPMI Medium 1640	n/a	To 500 mL

Store at 4°C for up to one month.

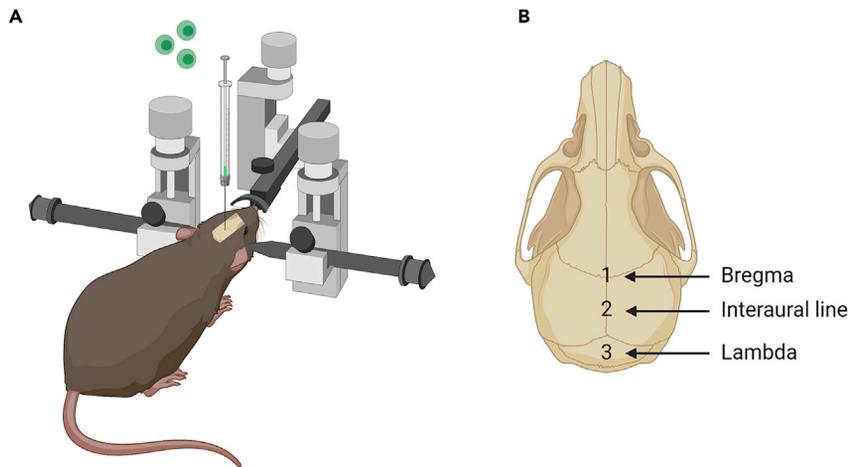


Figure 1. Schematic of ICV injection apparatus and mouse skull exposure

(A) Set-up of the mouse in the stereotaxic apparatus for the injection of CD4⁺ T cells.

(B) An exposed mouse skull showing the reference points, bregma (1), interaural line (2) and lambda (3), used for the correct positioning of the injection site.

STEP-BY-STEP METHOD DETAILS

ICV injection of CD4⁺ GFP T cells

⌚ **Timing:** 40–50 min/mouse

Note: This protocol describes the procedure for injection of one mouse.

1. Anesthetize the mouse in the induction chamber filled with 2% isoflurane.

⚠ **CRITICAL:** Confirm sufficient anesthesia by assessing the foot-pinch response.

2. Once the mouse is anesthetized, clean the head area with a cotton tipped applicator soaked in 70% ethanol, avoiding the eye area.
3. Using a scalpel, make a vertical incision down the center of the head, starting between the eyes, and use a cotton tipped applicator to expose the skull and dry the area.

Note: Be careful not to damage the skull with the scalpel.

4. Place the mouse in the stereotaxic apparatus (Figure 1A), positioning the nose in the anesthesia adaptor, with a constant flow of 2% isoflurane, and tighten the ear bar on the right-hand-side until the head is firmly in place.

Note: The position of the left-hand-side ear bar is set at the start of all the injections and should not be adjusted throughout the injections.

⚠ **CRITICAL:** Place mouse over the heating pad to prevent hypothermia during the procedure.

5. Lubricate the mouse's eyes with Viscotears Liquid Gel to prevent corneal dryness.
6. Insert the drill into the holder of the stereotaxic apparatus.

Note: Ensure the drill is correctly inserted into the holder. If not, the drill can move during drilling and damage the brain or drill the hole in the wrong area.

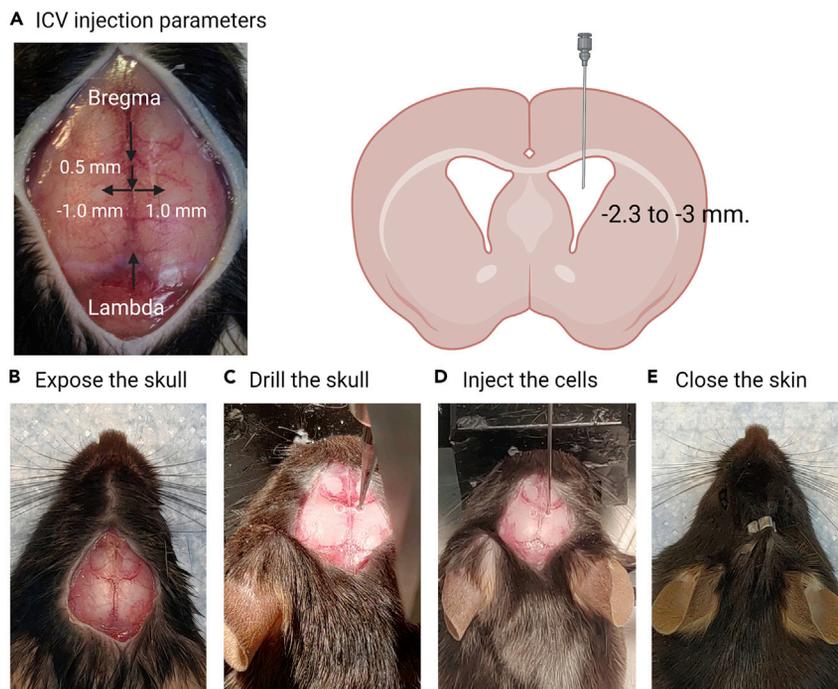


Figure 2. Outline of bilateral ICV injection procedure

- (A) ICV injection coordinates showing the reference points, bregma and lambda.
 (B) The skull is exposed by incising the skin.
 (C) Two holes are drilled in the skull, using the bregma and lambda as reference points.
 (D) The cells are ICV injected.
 (E) The skin is closed using a metal suture clip.

7. Using the drill, ensure the bregma and lambda are on the same horizontal plane. For this, gently place the tip of the drill at the bregma position. Set the dorsoventral axis to zero mm on the stereotaxic apparatus. Raise the tip of the drill and move directly down to the Lambda position. Lower the tip of the drill to the skull. If the dorsoventral axis is now ≤ 0.1 mm, the head of the mouse is straight. If not, adjust the position of the head and repeat until the bregma and lambda are on the same horizontal plane.

△ CRITICAL: Ensure the head is in the correct position and stable to achieve injection to the ventricles.

8. Once the head is straight, drill two small holes in the skull, just large enough for the needle to pass through and infiltrate the brain surface. Follow the coordinates measured from the bregma, to reach the lateral ventricles: -0.5 mm in the anteroposterior axis, ± 1 mm in the mediolateral axis and -2.3 mm in the dorsoventral axis (Figure 2A).

△ CRITICAL: Be careful not to cause brain damage.

Note: Stereotactic coordinates are optimized for 2-month-old mice and older (Allen Brain Atlas). The injection range for the dorsoventral axis is -2.3 to -3 mm. It is optimal to calibrate this coordinate for your specific mice prior to experiments, as it might change depending on the mouse strain and age. This can be achieved by injecting a dye at different coordinates in the dorsoventral axis. A successful injection is identified by the presence of the injected dye in the lateral ventricles.

9. Prepare cells for injection by adding 1 mL of PBS to a previously prepared aliquot. Centrifuge at 500 g for 5 min at 20°C. Remove the supernatant and suspend in 2.5 μ L PBS.

Note: Prior to experiment cells are prepared in aliquots and kept on ice.

10. Load the Hamilton syringe, holding either the 26 G or 30 G needle, with the desired treatment (here transduced CD4⁺ GFP T cells suspended in PBS) and place it in the arm of the stereotaxic device connected to an automated microinjection system. For control mice, inject 2.5 μ L of PBS or control cells suspended in PBS, to lateral ventricle of the brain.

△ CRITICAL: Ensure there are no air bubbles in the syringe.

11. Position the syringe over the first hole, lower the needle until it is touching the brain, and set the dorsoventral axis to zero mm.
12. Very slowly lower the needle to -2.3 mm in the dorsoventral axis.
13. Set the infusion pump to administer the injection at a rate of 1 μ L per minute.
14. Once the infusion is finished, wait for 2 min before raising the needle slowly to -1.15 mm in the dorsoventral axis. Then, wait 1 min before slowly raising the needle out of the hole.

△ CRITICAL: Always wait the recommended time before raising the needle and raise the needle slowly to minimize backflow.

15. Based on your experimental design, repeat the same procedure for the second lateral ventricle.
16. Close the incision using a stainless steel wound clip.

Note: It is recommended to use an appropriate analgesic post-injection, for example, buprenorphine 0.05 mg/kg subcutaneously.

17. Return the mouse back to the colony.
18. Monitor the mouse until it wakes up and moves around normally.

Preparation of brain sections for immunohistochemistry

⌚ **Timing:** 5–6 days

19. Sacrifice the mouse 72 h post injection.
20. Perfuse the mouse with 10 mL cold PBS.

Note: Perfusion was important for our experimental setup as we were interested in locating the brain-infiltrating injected cells and not the injected cells located within the brain vasculature.

21. Harvest the brain.
22. In a 15 mL centrifuge tube, suspend the brain in 4% paraformaldehyde for 48 h at 4°C.
23. Switch to 30% sucrose in PBS solution for 48 h, or until the brain has sunk to the bottom of the 15 mL centrifuge tube.

⏸ **Pause point:** The brain can be left immersed in 30% sucrose at 4°C for up to 5 days.

24. Embed in O.C.T. compound and freeze blocks at -20°C .

⏸ **Pause point:** O.C.T. blocks can be stored at -80°C for up to 6 months.

25. Using the microtome, cut brains to 40 μm sections.
26. In a 48 well plate, rinse the tissues in 0.5% Triton in PBS solution for 30 min.
27. Rinse tissue twice with PBST for 5 min.
28. Transfer the tissues to DAPI 1:3000 in PBS and incubate for 5 min.
29. Rinse the tissues with PBST for 5 min.
30. Transfer the tissue onto a slide. Once they have dried, add anti-fade fluorescence mounting medium and cover the sections with a cover slip.
31. Image brain sections on a FLUOVIEW FV1000 laser-scanning confocal microscope.

EXPECTED OUTCOMES

To optimize the ICV injection by means of cell migration into the site of action, CD4⁺ GFP T cells were ICV-injected to C57BL/6JRcchsd mice (Figure 1B). Mice were sacrificed 72 h post injection and sagittal brain sections were obtained for immunohistochemistry analysis. Brain sections were imaged using a FLUOVIEW FV1000 laser-scanning confocal microscope.

To rule out any influence of the needle gauge size on cell viability, CD4⁺ T cells were seeded (50,000 cells per well) in a 96 U-shape well plate. The cells were drawn into the syringe, holding either a 26 G or 30 G needle, twice. The live and dead cells were counted, using trypan blue to detect the non-viable cells, and the percent of live cells was calculated. The needle gauge size did not significantly affect cell viability (Figure 3B).

Although the needle gauge size had no significant effect on cell viability, an increased frequency of injected cells was observed in the brain parenchyma using the 30 G, as compared with the 26 G size needle (Figure 3C). Thus, while optimizing the injection procedure, we have concluded that 1) the 30 G needle size increases the frequency of the injected cells at the site of action, and 2) the smallest drill head suitable for the needle gauge size should be used to minimize the backflow.

LIMITATIONS

One of the main limitations of ICV injection is the lengthy time per mouse, making it laborious and time consuming. Depending on the facility and personnel availability, we suggest to adjust the cohort size performed per day. It is also essential to ensure that the mice are not away from the animal facility for a prolonged duration. The mice should have free access to food and water.

The syringe should be thoroughly washed with 70% ethanol, PBS and then clean PBS between injections to avoid cross contamination of treatments. The double PBS washing reduces the tendency for salts sticking to the inside of the syringe. The proper washing procedure also reduces the risk of excess fluid being held in the syringe and injected to the next mouse. It is also important to note that the cells should only be drawn into the syringe once. If cells are repeatedly drawn into and released from the syringe, cell viability may be reduced.

We advise using isoflurane as the anesthesia as it provides effective short-term anesthesia effects. It is possible to use an alternative anesthesia, such as a ketamine/xylazine regimen, but this comes with a prolonged recovery time and an increased risk of hypothermia. If ketamine/xylazine is preferred, mice should be closely monitored until they regain consciousness and move around by themselves. It is also recommended to provide heat, such as a heating pad, during the recovery time. A rectal thermometer can be used to monitor the body temperature during the procedure and recovery time.

As it may be necessary to divide the cohort over several days and the injection time of each batch of cells may take a few hours, it is essential to check the viability and homogeneity of the cell samples throughout the experiment. This can be done via cell quality validation with flow cytometry analysis performed on the day of injection.

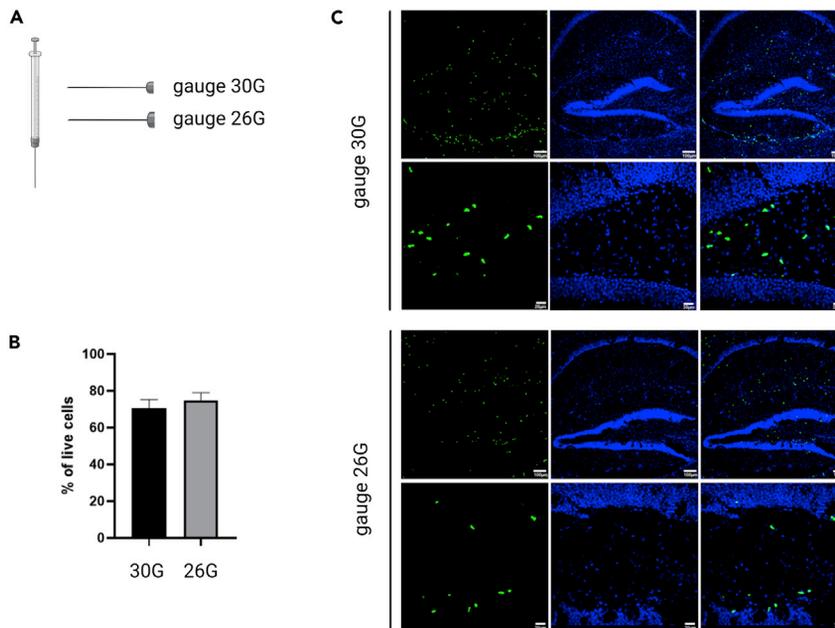


Figure 3. ICV injection of CD4⁺ T cells is optimized with the 30 G needle

(A) Schematic of a Hamilton syringe with a 26 G and 30 G needle.

(B) Percent of live cells using the 26 G or the 30 G needle. CD4⁺ GFP T cells were seeded in a 96 U-shape well plate (50,000 cells per well). Cells were drawn into the syringe, holding either a 26 G or 30 G needle, twice. Live and dead cells were counted and the percent of live cells was calculated. Error bars represent mean \pm SEM.

(C) Distribution of the ICV-injected CD4⁺ GFP T cells in hippocampal sections. C57BL/6JRcHsd mice were ICV-injected with 0.5×10^6 CD4⁺ GFP⁺ T cells per hemisphere. After 72 h, the mice were sacrificed, the brains sectioned, and the sections counterstained with DAPI. GFP (green) and DAPI (blue) were visualized by confocal microscopy. Upper panels show results for 26 G needle and lower panels show results for 30 G needle. Scale bar 20 μ m and 100 μ m.

TROUBLESHOOTING

Problem 1

Failure to correctly mount mouse onto stereotactic apparatus (steps 1 and 4).

Potential solution

Ensure the mouse is fully anesthetized before transferring it to the stereotactic apparatus.

Confirm sufficient anesthesia by assessing the foot-pinch response.

Set position of left ear bar at start of all the injections and only adjust the right ear bar to secure head in position.

Ensure the mouse's nose is securely in the anesthesia adaptor to ensure the mouse remains anesthetized throughout the procedure.

Problem 2

Failed injection due to incorrect injection site (step 6, 7 and 8).

Potential solution

Confirm you can clearly visualize the bregma and lambda.

Ensure the mouse head is mounted straight by utilizing the bregma and lambda as reference points.

Ensure the head is stable by gently pressing on the top of the head, once mounted. This ensures the head does not move during the drilling and injection steps.

Ensure the drill is inserted into the holder correctly.

Problem 3

Backflow of the injected cell suspension (steps 8 and 12–14).

Potential solution

Use the smallest drill head size suitable for the needle.

Inject at a flow rate of 1 $\mu\text{L}/\text{min}$.

Once the injection is finished, wait 2 min before slowly raising the needle to -1.15 mm in the dorso-ventral axis. Then wait a further minute before slowly raising the needle out of the hole.

Insert and raise the needle slowly to help decrease the backflow rate.

Problem 4

Bleeding due to incorrect drilling of the skull (steps 6 and 8)

Potential solution

Ensure the drill is inserted correctly into the holder. Otherwise, the drill can move during drilling resulting in the hole being drilled in the wrong area or damage to the brain.

It is important not to drill too deep to prevent puncturing the skull resulting in bleeding and inflammation of the brain.

If bleeding does occur due to drilling, use a gauze to apply pressure to the injection site until the bleeding has stopped.

If brain is badly damaged, exclude the mouse from the experiment.

Problem 5

Death of mouse (steps 1, 4, and 18).

Potential solution

Ensure to use the correct isoflurane concentration, we use 2%.

It is also possible to use alternative anesthesia, for example, ketamine xylazine, but recovery time is longer and comes with a higher risk of hypothermia.

Place mouse on heat pad during procedure to prevent hypothermia.

Monitor mice post procedure until they wake up and are moving on their own.

Problem 6

Decreased viability of cells if left on ice too long (step 9).

Potential solution

If many mice need to be injected, prepare the cells in different batches throughout the day to prevent them being on ice for too long.

Depending on your experimental needs, it may be an option to decrease the injection time per mouse by injecting unilaterally.

Prepare aliquots of cells in culture media, only wash and suspend in PBS directly prior to injection.

Ensure to sufficiently clean the syringe with ethanol 70% and PBS between injections.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ekaterina Eremenko (eremenkoem@gmail.com).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This protocol did not generate/need datasets.

ACKNOWLEDGMENTS

This research was supported by funding from The National Institute for Biotechnology in the Negev Ltd., and The Ministry of Science and Technology (grant # 3-16148). The graphical abstract and [Figures 1 and 3](#) were created with [BioRender.com](https://www.biorender.com).

AUTHOR CONTRIBUTIONS

Z.V.T., E.E., and B.K. performed the experiments. Z.V.T., E.E., A.P., and A.M. designed the experiments and wrote the manuscript. All authors read, revised, and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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