In vitro Visualization of Cell-to-Cell Interactions between Natural Killer Cells and Sensory Neurons

Running title: NK cell & sensory neuron co-culture

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In vitro Visualization of Cell-to-Cell Interactions between Natural Killer Cells and Sensory Neurons

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Abstract

Cell-to-cell interactions between the immune and nervous systems are increasingly recognized for their importance in health and disease. Assessment of cellular neuro-immune interactions can be aided by co-culture of two (or more) cells in an *in vitro* model system that preserves the morphology of neuronal cells. Here we describe methods to investigate the cytotoxic effector functions of natural killer cells on sensory neurons isolated from syngeneic embryonic and adult mice. We present methods for the morphological analysis of axon fragmentation (pruning) and dynamic cell function via live confocal calcium imaging. These techniques can easily be adapted to study interactions between other combinations of immune cell subsets and neuronal populations.

Key words: Axon fragmentation, Calcium imaging, Cellular cytotoxicity, Confocal microscopy, Magnetic Cell Sorting, Immune synapse.

1 Introduction

Cytotoxic immune cells, including natural killer (NK) cells, are now known to play an important role in disorders of both the central and peripheral nervous systems [1, 2]. In the brain and spinal cord, NK cell function may be a driver of neurodegeneration by targeting neuronal precursor cells in the aged brain

[3], and killing of motor neurons by direct neurotoxicity or indirectly via pro-inflammatory cytokine release in a model of amyotrophic lateral sclerosis (ALS) [4]. On the other hand, NK cells may be beneficial in Parkinson's disease via their ability sequester α synuclein [5] as well as in multiple sclerosis by controlling T cell activity [6]. In the peripheral nervous system, the pruning of injured sensory axons by NK cells has been suggested to help resolve neuropathic pain [7]. To understand the importance of these varied immune effector functions on the nervous system, NK cells must be studied as they physically interact with neurons and other cellular partners. Combining co-cultures with live imaging also allows for real time visualization of the neuron-immune synapse [1]. Here we describe the isolation and co-culture of mouse dorsal root ganglia (DRG) sensory neurons with syngeneic splenic NK cells, and provide a detailed explanation of methods that were employed in our previous study [7]. DRG neurons can be cultured on glass bottom dishes or microfluidic chambers and provide a robust neuronal substrate for the study of cytotoxicity and other neuro-immune interactions.

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2 Materials

2.1 Common equipment

1. Personal protective equipment (nitrile gloves, laboratory coat, goggles).
2. Water bath set to 37°C.
3. Cell culture incubator (5% CO_2, humidified atmosphere).
4. Alcohol lamp for fire-polishing glass Pasteur pipettes.
5. Centrifuge with capacity for 15 ml and 50 ml conical tubes.
6. Inverted phase-contrast microscope.

7. Hemocytometer.
8. Pipettes & sterilized tips.
9. Pipette aid & disposable pipettes.
10. 35 & 100 mm Petri dish.
11. 15 & 50 ml conical tubes.
12. 1.5 ml sample tubes.
13. Basic rodent surgical tools (cleaned with 70% ethanol and air dried).
14. Ice.
15. Vacuum pump aspirator.
16. Plasma cleaner.
17. Microbiological safety cabinet (MSC).

2.2 Primary NK cell isolation and stimulation

1. Mouse NK cell isolation kit II (Cat# 130-096-892, Miltenyi Biotec).
2. MACS Separator (Cat# 130-042-302 or 130-090-976, Miltenyi Biotec).
3. MACS Multi-stand (Cat# 130-042-303, Miltenyi Biotec).
4. LS columns (Cat# 130-042-401, Miltenyi Biotec).
5. Clamp stand.
6. Nylon wool fiber (Cat# 18369, Polysciences).
7. ACK lysis buffer: Distilled water, 150mM NH_4Cl, 10mM $KHCO_3$, 0.1mM EDTA. Adjust to pH 7.3. Autoclave and store buffer at room

temperature.

8. Magnetic-activated cell sorting (MACS) buffer: 0.01M Phosphate buffered saline (PBS), 2 mM EDTA, 2% (v/v) FBS. Autoclave before adding sterile FBS.

9. RPMI/FBS: RPMI medium 1640 (Cat# 11875-093, Gibco), 10% (v/v) FBS, 1% (v/v) Penicillin-Streptomycin.

10. Recombinant murine interleukin 2 (IL-2) (Cat# 212-12, Peprotech). First, reconstitute at 10^6 U/ml in distilled water and incubate at room temperature (15 min), before diluting further in PBS containing 0.1% bovine serum albumin (BSA) to a final concentration of 10^5 U/ml. Store single-use aliquots at -80°C (*see* **Note 1**).

11. Cell strainers. 70 and 40 μm cell strainer (Cat# 352350 and 352340, Falcon).

12. 10 ml syringe.

13. 3-way plastic stopcock.

14. 96-well U-bottom culture plates (Cat# 353077, Falcon).

2.3 Primary DRG neuron culture

1. HBSS/HEPES: Calcium & magnesium-free Hank's Balanced Salt Solution (HBSS), 20 mM HEPES. Filter and autoclave before use. Store at 4°C.

2. Collagenase/Dispase solution (C/D): HBSS, 1 mg/ml Collagenase A (Cat# 10 103 578 001, Roche), 2.4 U/ml Dispase II (Cat# 04 942 078 001,

Roche). Filter sterilize (0.2 μm), aliquot and store at -20°C.

3. Trypsin solution: HBSS, 0.25% Trypsin. Store aliquots at -20°C.
4. Trypsin inhibitor: PBS, 2.5 mg/ml trypsin inhibitor (Cat# T9003, Sigma). Filter sterilize (0.2 μm), aliquot and store at -20°C.
5. DNase I: PBS, 2,500 U/ml DNase I. Aliquot and store at -20°C.
6. Dubellco's Modified Eagle's Medium (DMEM) (Cat# 11995-065, Gibco).
7. DMEM/FBS media: DMEM, 10% FBS, 1% Penicillin-Streptomycin.
8. DMEM/BSA: DMEM, 15% BSA. Mix 30% BSA solution (Cat# A6003, Sigma) with equal volume of serum-free DMEM.
9. Neurobasal/B27: Neurobasal medium (Cat# 21103-49, Gibco), 1X B27 (Cat# 17504-044, Gibco), 1mM L-glutamine (Cat# G7513, Sigma).
10. Nerve growth factor (NGF). Prepare mouse NGF 2.5S (Cat# 13257-019, Gibco) at 50-100 μg/ml in sterile distilled water. Aliquot and store at -20°C.
11. Poly-D-lysine (PDL) solution. Dilute PDL to 10 μg/ml in sterilized water before use. Aliquot 0.1-1 mg/ml stock and store at -20°C.
12. Laminin solution. Dilute laminin at 10 μg/ml in PBS. The products are recommended to be aliquot and kept in -20°C.
13. Glass Pasteur pipettes. Autoclave before use.
14. Glass bottom dishes (for example, Cat# 12-567-400, Thermo Scientific).
15. Two-compartment microfluidic devices constructed of

polydimethylsiloxane (PDMS) containing 3 µm x 10 µm x 500 µm channels between soma and neurite chambers were used for experiments (Cat# SND450, Xona Microfluidics).

2.4 Co-culture and live-cell imaging

1. Vybrant DiI (Cat# V22886, Molecular Probes).
2. Rhod-3 Calcium Imaging Kit (Cat# R10145, Molecular Probes).
3. CFSE Cell Proliferation Kit (Cat# C34570, Molecular Probes).
4. Confocal microscope (e.g. LSM 700, Zeiss).

2.5 Immunocytochemistry

1. Paraformaldehyde (PFA): 0.01M PBS, 4% PFA, pH 7.4. Prepare fresh when needed.
2. Blocking solution: PBS, 5% normal serum, 0.1% triton X-100.
3. Rabbit anti-β-tubulin III (Cat# T2200, Sigma).
4. Goat anti-NKp46 (Cat# AF2225, R&D Systems).
5. VECTORSHIELD HardSet Anti-fade Mounting Medium DAPI (Cat# H-1500, Vector).
6. ImageJ software. Image Processing and Analysis in Java (https://imagej.nih.gov/ij/).

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3 Methods

3.1 Primary NK cell isolation and stimulation

3.1.1 Preparation day before the experiment

1. Tease apart by hand 0.60 g of nylon wool fiber per column (*see* **Note 2**).
2. Remove plunger from 10 ml syringe and loosely pack with the teased nylon fiber.
3. Loosely wrap the syringe with aluminum foil and then sterilize by autoclave. Dry overnight in oven at approximately 55°C.

3.1.2 Preparation day of the experiment

1. In MSC, affix a 3-way stopcock to the sterile 10 ml nylon wool column.
2. Fasten the column in a clamp stand and place a tube or beaker underneath to collect the flow-through.
3. Pass 5 ml MACS buffer through the column at least 2-3 times for washing and wetting.
4. Carefully compress the nylon wool fiber to the bottom of the column using a serological pipette. Make sure the nylon wool is saturated with MACS buffer and no air bubbles remain.
5. Close the stopcock and add 2-3 ml of MACS buffer.
6. Release the column from clamp and carefully seal the column with aluminum foil to avoid contamination.
7. Incubate at 37 ℃ and get ready for spleen dissection.

3.1.3 Spleen dissection

1. Prepare 13 ml RPMI/FBS in 15 ml tube on ice.
2. Adult mice are killed by inhalation of a rising lethal concentration of isoflurane or carbon dioxide, and confirmed by cervical dislocation in accordance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 (*see* **Note 3**).
3. Remove connective tissue from the spleen using fine scissors.
4. Place the spleen inside 15 ml tube containing RPMI/FBS on ice and proceed immediately to next step.

3.1.4 Isolation of the splenocytes

1. Place 70 μm cell strainer on a petri dish and moisten the strainer with RPMI/FBS.
2. Place 40 μm cell strainer on a 50 ml tube.
3. Transfer a spleen onto the 70 μm cell strainer using forceps and apply 1 ml RPMI/FBS to the tissue.
4. Mash the spleen gently using the syringe plunger flange to create a single cell suspension. Repeat this procedure with additional washes with RPMI/FBS.
5. Collect the suspension from petri dish and transfer to the 50 ml tube passing through the 40 μm strainer.
6. Repeat step 3 to 5 with remaining spleens and collect in one 50 ml tube.
7. Pellet the cells at 400 x g for 5 min. Aspirate the supernatant.

8. Loosen the pellet by flicking the end of the tube, then add 1 ml of ACK lysis buffer per spleen. Gently re-suspend the cells and incubate at room temperature for exactly 2 min to lyse erythrocytes.
9. Directly add 30 ml of RPMI/FBS to arrest lysis and centrifuge at 400 x g for 5 min.
10. Aspirate the supernatant entirely. Re-suspend the pellet with 2 ml RPMI/FBS per maximum 3 spleens (per one nylon wool column) for the next steps.

3.1.5 NK cell isolation and IL-2 stimulation

1. Fit the pre-incubated nylon wool column to the clamp stand and wash the column several times with MACS buffer pre-warmed to 37°C.
2. Close the stopcock and overlay the 2 ml of splenocyte suspension on top of the nylon wool (**Fig. 1a**). Carefully open the stopcock to allow the suspension to percolate into the nylon wool. Once percolated, close the stopcock to prevent loss of cells (**Fig. 1b**).
3. Add an additional 2 ml of pre-warmed MACS buffer to protect from drying (**Fig. 1c**).
4. Carefully release the column from the clamp and seal with aluminum foil.
5. Incubate the column for 1 h at 37 °C.
6. Reinstall the column to the clamp stand and place a 50 ml tube underneath for each column.
7. Collect the flow-through in a 50 ml tube by opening the stopcock.

8. Pass 5ml pre-warmed MACS buffer through the column and collect the flow-through in the same tube. Repeat this procedure until a total of 30 ml of buffer is collected in the tube.
9. Count the cells to decide the amount of the buffer for resuspension.
10. Pellet the cells at 300 x g for 10 min. Remove supernatant.
11. Gently re-suspend the cells with 40 µl MACS buffer per 10^7 cells.
12. Directly add 10 µl of biotinylated antibody cocktail (Mouse NK cell isolation kit II) per 10^7 cells. Mix gently and incubate at 4℃ for 5 min.
13. After incubation, directly add 2 ml of MACS buffer per 10^7 cells for washing.
14. Pellet the cells at 300 x g for 10 min. Remove supernatant. Gently re-suspend the cells with 80 µl MACS buffer per 10^7 cells.
15. Directly add 20 µl of streptavidin beads (Mouse NK cell isolation kit II) per 10^7 cells. Mix gently and incubate at 4℃ for 10 min.
16. Prepare MACS stand, separator and LS columns for next step. One LS column is used for a maximum of 10^8 cells.
17. Pass the cells and antibody mixture through the LS column and collect in 15 ml tube.
18. Add an additional 3 ml of MACS buffer to the LS column and collect in the same tube. Cells that pass through will be unlabeled NK cells.
19. Adjust to 10 ml with MACS buffer and count the cells to decide the

amount of buffer for resuspension.

20. Pellet the purified NK cells at 300 x g for 10 min (*see* **Note 4**). Remove supernatant.
21. Add RPMI/FBS pre-warmed to 37C° to a final density of $2x10^6$ cells per 1 ml.
22. Add 1000 U of IL-2 (10 µl of the 10^5 U/ml IL-2 stock) per $2x10^6$ cells (1 ml of the cell suspension).
23. Seed $4x10^5$ cells (200 µl of the cell suspension) per well in a U-bottom culture plate.
24. Incubate at 37 ℃ incubator for 2 days for NK cell stimulation.

3.2 Primary DRG neuron culture

3.2.1 Preparation of glass bottom dishes

1. Coat the glass area with 10 µg/ml PDL in distilled water (100 µl per dish) overnight at room temperature (*see* **Note 5**).
2. Next day, aspirate PDL and wash the dishes with distilled water several times. Leave to dry for 1 h in MSC.
3. Coat with 10 µg/ml laminin in PBS (100 µl per dish) and incubate at least for 1 h in MSC.
4. Remove the laminin solution immediately before seeding DRG neurons. Laminin-coated dishes are to be used on the day.

3.2.2 Preparation of microfluidic chambers

1. Attach the microfluidic devices to glass coverslips after treatment in a plasma cleaner.

2. Immediately after plasma-bonding, coat the reservoirs overnight with 10 µg/ml PDL followed by washing with distilled water and aspirate excess water from reservoirs. Be careful not to remove any fluid from microfluidic channels.

3. Coat with 10 µg/ml laminin in PBS and incubate at least for 1 h in MSC.

4. Remove the laminin solution immediately prior to DRG neuron seeding.

3.2.3 Adult mouse DRG dissection

1. Adult mice are killed by inhalation of a rising lethal concentration of isoflurane or carbon dioxide, and confirmed by cervical dislocation in accordance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986 (*see* **Note 6**).

2. Bilateral DRG are rapidly dissected on ice-cold HBSS/HEPES.

3. Spinal nerves and roots are trimmed and dural membranes removed using fine forceps under a dissection microscope (*see* **Fig. 2**).

4. Dissected DRG are carefully transferred with forceps to a 15ml tube containing HBSS/HEPES and centrifuged at 200 x g for 3 min and supernatant discarded.

3.2.4 Embryonic mouse DRG dissection

1. A timed mated female mouse carrying pups at embryonic day 15 (E15) is killed by inhalation of a rising lethal concentration of isoflurane or carbon

dioxide, and death confirmed by cervical dislocation (*see* **Note 6**).

2. Spray the abdomen of the mouse with 70% ethanol and make an incision to remove the embryonic sac to a petri dish of HBSS/HEPES on ice. Rapidly remove individual embryos from the sac.

3. To dissect DRG, decapitate the embryos and make an incision down the spine using fine spring scissors.

4. Remove the spinal cord to expose the bilateral DRG. DRGs are collected using fine, mirror finished forceps directly to a 15 ml tube of HBSS/HEPES on ice.

5. Once all DRG from all pups are collected, centrifuge tissue at 200 x g, 5 min (*see* **Note 7**).

3.2.5 DRG neuron enzymatic dissociation

1. Pre-warm all the media used for the DRG dissociation steps to 37°C.

2. Digest the isolated DRGs for 30 min (embryonic) to 60 min (adult) in collagenase/dispase solution at 37°C, centrifuge at 200 x g for 3 min and remove the supernatant (*see* **Note 8**).

3. Carry out an additional digestion for 5-7 min in 2ml of 0.25% trypsin in HBSS at 37°C and stop with an equal volume of 2.5 mg/ml trypsin inhibitor solution. Add another volume of DMEM/FBS before centrifugation at 200 x g for 3 min and discard the supernatant. Repeat the wash with DMEM/FBS once.

4. Re-suspended the DRGs in 1.8 ml DMEM media only (serum-free)

supplemented with 125U/ml DNase I and dissociate DRGs by gentle trituration through a large bore fire-polished glass Pasteur pipette for 5-10 times (*see* **Note 9**).

5. Allow the cell suspension to settle for 1 min and remove the upper half volume containing dissociated cells to a new 15 ml tube.

6. Add 0.9 ml of fresh DMEM media to the original tube and dissociate the rest of the DRG tissue by gentle trituration through a narrow bore glass Pasteur pipette for 3-4 times. Repeat steps 5 & 6 until no intact DRG remain.

7. Collect all of the cell suspension and then carefully lay over at DMEM/BSA (*see* **Note 10**). The volume of BSA solution should be twice the volume of the cell suspension.

8. Centrifuge the suspension at 200 x g for 10 min using slow acceleration and minimum brake.

9. Carefully remove the overlying media, interface (containing myelin debris) and DMEM/BSA using an aspirator to leave the DRG cell pellet (*see* **Fig. 3**).

10. Re-suspend DRG neuron pellet in pre-warmed Neurobasal/B27 supplemented with 50 ng/ml NGF.

3.2.6 Seeding DRG neurons on glass bottom dishes

1. Count the number of live DRG neurons using a hemocytometer and trypan blue for exclusion of dead cells.

2. Dilute DRG neurons to a density of 10^4 per ml in Neurobasal/B27 supplemented with 50 ng/ml NGF.

3. Aspirate excess laminin solution from the glass bottom dish immediately prior to seeding (*see* **Note 11**).

4. Seed 10^3 adult or $8x10^3$ embryonic DRG neurons in total volume of 100 µl per a dish (*see* **Note 12**).

5. Incubate at 37°C for 1 h to allow cells to attach and then add additional 100 µl of Neurobasal/B27 plus 50 ng/ml NGF to the glass area (*see* **Fig. 4a, b**).

3.2.7 Seeding DRG neurons in microfluidic devices

1. Dilute DRG neurons to a density of $2x10^5$ per ml in culture media containing 100 ng/ml NGF.

2. For microfluidic devices, aspirate excess laminin solution from cell soma and neurite reservoirs being careful not to draw any fluid from the microfluidic channels.

3. Seed 10^4 DRG neurons in a volume of 5 µl directly into the soma channel of the microfluidic device (*see* **Fig. 4c**).

4. Incubate at 37°C for 1 h to allow cells to attach before flooding the four reservoirs with Neurobasal/B27.

5. Place microfluidic chambers in appropriately sized petri dishes (e.g. 35 mm diameter) preferably with low-evaporation lids and incubate at 37°C, 5% CO_2.

6. Change Neurobasal/B27 every day to maintain neuronal health and prevent evaporation. The addition of 100 ng/ml NGF to Neurobasal/B27 in the neurite reservoir encourages neurite outgrowth (*see* **Note 13**).

3.3 NK cell and DRG neuron co-culture

3.3.1 Fluorescent labelling of live NK cells and DRG neurons.

1. Cultured adult or embryonic mouse DRG neurons can be fluorescently labelled with Vybrant DiI (Ex 549 nm/Em 565 nm) or loaded with the Ca^{2+} indicator rhodamine 3-AM (Ex 560 nm/Em 600 nm) (Rhod-3 Calcium Imaging Kit). NK cells with or without IL-2 stimulation can be labelled with CFSE (Ex 492 nm, Em 517 nm) before co-culture for visualization by fluorescence imaging (*see* **Note 14**).

2. For Vybrant DiI labeling of DRG neurons, prepare staining medium by adding Vybrant DiI to Neurobasal/B27 at 1:200 dilution. Replace the culture media with the staining media and incubate the cells at 37°C for 20 min. Wash the cells with fresh pre-warmed 37°C Neurobasal/B27 (3 x 10 min incubation).

3. For Rhod-3 AM loading, prepare loading medium by adding 10 μM Rhod-3 AM stock, 1X PowerLoad concentrate and 2.5 mM Probenecid (Rhod-3 Calcium Imaging Kit) to Neurobasal/B27. Replace the culture media with the loading media and incubate the cells at 37°C in dark for 30-60 min. Wash the cells once with fresh pre-warmed 37°C Neurobasal/B27.

4. For CFSE labeling of NK cells in suspension, prepare labeling medium by adding 5 μM CellTrace CFSE dye in PBS. Replace the culture media

by centrifugation and re-suspension. Incubate the cells with labeling media at RT or 37°C in dark for 20 min. Dilute the media by adding five volumes of RPMI/FBS and incubate for 5 min to remove remaining free dye in the solution. Re-suspend the cells with fresh, pre-warmed 37°C RPMI/FBS following centrifugation. Incubate the cells at least for 10 min before co-culture.

3.3.2 Seeding NK cells on glass bottom dishes

1. Collect the enriched NK cells in a 15 ml tube. Wash with fresh pre-warmed 37°C RPMI/FBS at 300 x g for 10 min.
2. Count the cells and re-suspend at $2.5x10^6$ cells per ml in Neurobasal/B27 *without* NGF.
3. Carefully replace the DRG media with 100 µl of pre-warmed fresh Neurobasal/B27 *without* NGF.
4. Gently add $2.5x10^5$ NK cells (100 µl of NK cell suspension) dropwise on top of the DRG on glass bottom dishes, resulting in NK cell effector to adult DRG target ratio of 250:1, and an embryonic DRG target ratio of approximately 30:1.
5. Incubate at 37 °C for 4-6 h.

3.3.3 Seeding NK cells in microfluidic devices

1. For microfluidic cultures, gently remove the media from both reservoirs of the neurite channel.
2. Seed $1.25x10^5$ NK cells in 5 µl neuronal media directly to the neurite

channel (*see* **Fig 4d**). Do not add further media to reservoirs as this will wash the NK cells from the channel.

3. Incubate at 37 °C for 4-6 h.

3.3.4 Live imaging of glass bottom dishes

1. Immediately transfer the dishes containing neuron-NK co-cultures to a confocal microscope for live imaging (*see* **Note 15**).

2. Acquire a time series of single z-section images (512 x 512) using a multitrack setting (488nm and 555nm fluorescence emission and differential interference contrast (DIC) bright-field at 1 min intervals and multiple positions under the control of Definite Focus (Zeiss LSM 700) (*see* **Note 16**).

3. Images can be exported as a sequential time-lapse in AVI format.

4. Calcium responses can be quantified in rhodamine 3 neurons by counting 'flashes' in individual axons or cell bodies per frame/unit of time (*see* **Note 17**)

3.3.4 Fixed cell imaging (Immunocytochemistry)

1. After 4-6 h in co-culture, gently remove excess culture media and carefully wash cells three times with HBSS pre-warmed to 37°C.

2. Fix the cells by incubating 4% paraformaldehyde in PBS for 30 min at room temperature.

3. After washing in PBS (3 x 10 min), apply blocking solution for 1 h at room temperature.

4. Remove the blocking solution and apply primary antibodies in a 1 in 10 dilution of blocking solution in PBS overnight at 4°C in a humidified chamber (β-tubulin III for staining the neurons and NKp46 for NK cells) (*see* **Note 18**).

5. After washing primary antibodies in PBS (3 x 10 min), apply appropriate fluorescent conjugated secondary antibodies and incubate for 1 h at room temperature.

6. After washing in PBS, flood the dishes with PBS containing 0.02% sodium azide as a preservative.

7. Dishes may also be mounted with a coverslip and aqueous mounting media.

8. Fluorescence images (1024 x 1024 resolution) of 488 and 555 laser channels are acquired sequentially on a laser-scanning confocal microscope. Acquire z-section(s) using low magnification (x20 lens) confocal image at 0.5 digital zoom (*see* **Fig. 4b**).

9. Acquire images of 5-7 fields of view from each coverslip. To reduce sampling bias, perform region selection unsupervised or use tile scan or equivalent automated sampling function.

3.4 Analysis of neurite fragmentation

3.4.1 Semi-automated analysis using ImageJ

1. Semi-automated analysis is performed using ImageJ [8].

2. Neurite fragments are selected using the *Analyze Particles* function (size

0.5-75 μm^2, circularity 0-1) and saved as a drawing (**Fig. 5**).

3. The total area and particulate area values (μm^2) are obtained using the *Measure* function and used to calculate the percent neurite fragmentation for each field of view (*see* **Note 19**).

3.4.2 Image J macro

1. The speed of data processing can be greatly increased through the use of a macro script (*see* **Figure 6**) (*see* **Note 20**).
2. To create the macro file go to: ImageJ >> Plugins >> Macros >> Record...
3. Enter the macro script into the macro recording window, and save as an .ijm file.
4. To run the macro file go to: ImageJ >> Plugins >> Macros >> Run...
5. Select the .ijm file in the appropriate directory and the macro will run, asking for the Input directory containing .lsm files to analyse, and an Output directory in which to save the *Particle Analysis* drawings (*see* **Note 21**).

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4 Notes

1. Be sure to follow manufacturer's instructions for cytokine reconstitution. Incorrect reconstitution will reduce cytokine activity and result in suboptimal stimulation of murine NK cells.

2. One nylon wool column is for maximum 3 spleens. Fully disentangle the wool fibers for better separation of adherent cells (e.g. B cells, myeloid cells) and efficient flow-through of non-adherent lymphocytes.

3. Further guidance on methods of humane killing can be found at avma.org [9]. DRG neurons can be collected from the same mice and cultured for use at two days *in vitro*, reducing the need for additional animals.

4. Confirm purity of NK cells (CD3negNKp46+ cells, or NK1.1+CD49b (DX5 clone) double positive cells) by flow cytometry. We have found the mouse NK isolation kit II achieves >90 % purity of unlabeled NK cells despite omitting the nylon wool step, however the efficiency of depletion of adherent populations (e.g. B cells, myeloid cells) may differ. An alternative NK isolation is available from Stem Cell Technologies (EasySep, Cat# 19855).

5. The PDL concentration can be increased up to 100 μg/ml in case of weak adherence of neurons to the glass.

6. Embryonic DRG cultures are typically obtained from pregnant female mice at E15. This provides the optimum maturity to remove the DRG before the surrounding tissues harden, making dissection more difficult.

7. Tap the tube gently on the ice box to encourage the DRG to detach from the side of the tube and descend to the bottom before the centrifugation.

8. The mice aged 4-7 weeks are recommended for use. Different concentration of collagenase/dispase solution (0.2 mg/ml Collagenase A, 3.3 U/ml Dispase II) may be required for obtaining healthy neurons from

the mice older than 7 weeks.

9. Not all debris will completely dissociate after trituration with the fire-polished glass pipette (large-sized pore). Try to keep the number and velocity of triturations to a minimum to preserve DRG neuron viability.

10. Gradient centrifugation using a DMEM/BSA cushion will help remove myelin debris and dead cells. Ensure BSA and DMEM media are mixed thoroughly to guarantee gradient separation.

11. Make sure the coverslips do not dry after aspiration of laminin.

12. This number of neurons provides the optimum density for quantifying neurite fragmentation. Culture density may need adjustment depending on the aims of the experiment. Glass bottom dishes are ideal for live imaging using confocal, two-photon, spinning disc or deconvolution microscopy with a suitable holder and environmentally controlled chamber.

13. Adult DRG neurites take approximately 5 days to fully extend into the neurite chamber of the microfluidic device (**Fig. 4e**).

14. These dyes are suitable for live imaging but not for imaging after fixation.

15. Although cytotoxicity of NK cells against sensory neurons occurs within minutes of co-culture, use of a humidified chamber to maintain the atmosphere at 37°C and 5% CO_2 will ensure optimal cell function during recordings of up to 4 h or more.

16. Reduce confocal laser power as much as possible to reduce possible photo

bleaching. Signal intensity may be compensated by increasing the gain of each photomultiplier tube (PMT).

17. Rhodamine 3-AM is not a ratiometric dye. Absolute fluorescence intensity will depend on loading conditions and provides an indication of the relative change in intracellular calcium only.

18. Use a normal serum matching the species of the secondary antibody. The recommended dilutions for primary antibodies are: Rabbit anti-β-tubulin III (1: 500-1,000); goat anti-NKp46 (1:200).

19. In addition to fragmentation analysis of microfluidic cultures, the Plot Profile function in Image J can also be used to determine the horizontal pixel density as a measure of neurite die-back.

20. This macro script is compatible with ImageJ on a PC running Windows 10. Use of the macro with a Mac-based operating system may require additional formatting of the script.

21. The macro assumes that channel 'C2' is of interest. Threshold values will need to be adjusted according to the image acquisition settings used to ensure neuron morphology is adequately defined (*see* **Fig 5**).

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Figure legends

Fig. 1 Isolation of non-adherent lymphocytes using nylon wool column purification. **(a)** Splenocyte suspension laid over the nylon wool. **(b)** Percolation of the cell suspension into the nylon wool. **(c)** Addition of MACS buffer on top of the wool to protect from drying.

Fig. 2 Adult DRG tissue dissection. **(a)** Before and **(b)** after trimming of spinal nerves and roots. Images acquired on a dissection microscope at x4 magnification.

Fig. 3 The effect of DMEM/BSA gradient centrifugation to remove debris and non-neuronal cells. **(a)** Image of 15% BSA cushion after centrifugation. **(b)** A fine cell pellet may be visible after centrifugation (white arrow). Acutely cultured DRG neurons without **(c)** or with **(d)** BSA centrifugation. Scale bar, 100 μm. Note the reduction in myelin debris after DMEM/BSA centrifugation (**e**) DRG neurons after 48 h in culture without DMEM/BSA centrifugation. **(f)** DMEM/BSA centrifugation reduces the carry-over of non-neuronal cells (bright DAPI (1:5,000) nuclei), including Iba-1 (1:400, Wako) positive macrophage (asterisks). Scale bar, 50 μm.

Fig. 4 Formats of DRG neuron culture. **(a)** Schematic of glass bottom dish. **(b)** β-tubulin III immunofluorescence of adult DRG neurons 24 h after culture in glass bottom dish. Scale bar, 50 μm. **(c)** Schematic of two compartment microfluidic device. **(d)** Cell body chamber (left) and neurite chamber (right) to which IL-2 stimulated NK cells were added and incubated for 4h (β-tubulin III, magenta; NKp46, green). Note NK cells are capable of motility through the microfluidic channels. Scale bar, 100 μm. **(e)** Exported b/w image composite of β-tubulin III immunofluorescence of adult DRG axons 5 days after culture in a microfluidic chamber. Arrow indicates direction of neurite growth. Scale bar, 200 μm.

Fig. 5 Analysis of neurite fragmentation. **(a)** Threshold image of DRG neurons cultured in the absence of NK cells. **(b)** Selection for measurement of total area in (a). **(c)** Neurite fragments from (a) after

running *Analyze Particle* function. **(d)** Threshold image of DRG neuron after 4 h co-culture with NK cells. **(e)** Selection for measurement of total area in (d). **(f)** Neurite fragments from (d) after running *Analyze Particle* function. Scale bars, 100 µm.

Fig. 6 This macro is designed to perform particle analysis on a batch of raw .lsm confocal images of sensory neurons acquired with three fluorescence channels and two or more z-sections.

Figure 1

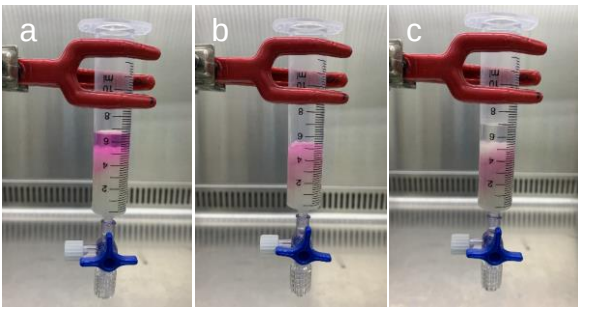

Figure 2

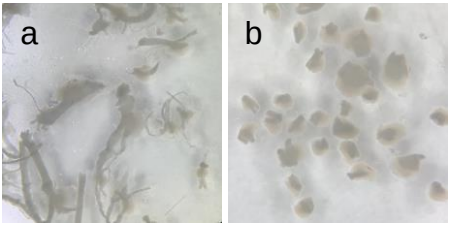

Figure 3

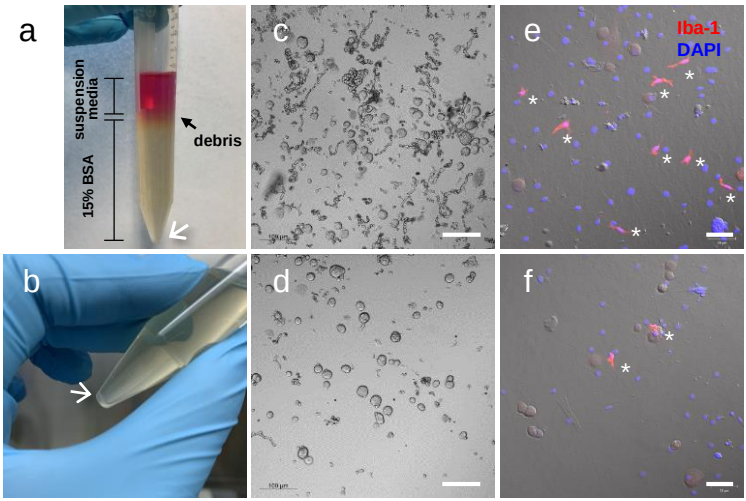

Figure 4

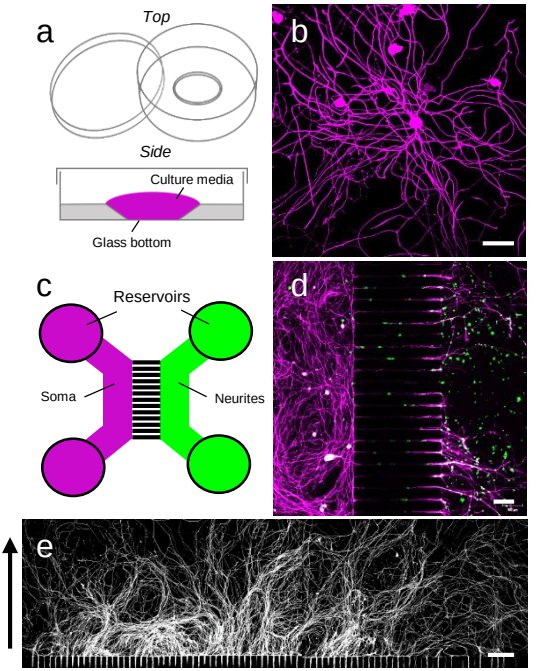
a
Top
Side
Culture media
Glass bottom
b
c
Reservoirs
Soma
Neurites
d
e

Figure 5

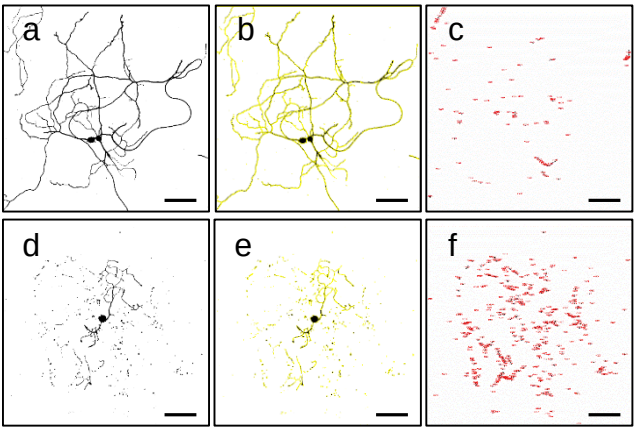

Figure 6

```
//SELECT dir AND GET IMAGES:

dir = getDirectory("select input dir");
list = getFileList(dir);

dir2 = getDirectory("select output dir");

for(a=0; a<list.length; a++) {

    open(dir+list[a]);
    wait(200);
    imageTitle=getTitle();
    run("Z Project...", "projection=[Max Intensity]");
    wait(200);
    run("Split Channels");
    wait(200);
    selectWindow("C2-MAX_" +imageTitle);
    setAutoThreshold("Default dark");
    //run("Threshold...");
    setThreshold(1209, 4095);
    setOption("BlackBackground", false);
    run("Convert to Mask");
    run("Analyze Particles...", "size=0.5-75 circularity=0-1.00 show=Outlines summarize");
    selectWindow(imageTitle);
    close();
    selectWindow("C2-MAX_" +imageTitle);
    close();
    selectWindow("C3-MAX_" + imageTitle);
    close();
    selectWindow("C1-MAX_" + imageTitle);
    close();
    selectWindow("Drawing of "+"C2-MAX_" +imageTitle);

    imageTitle=getTitle();
    saveAs("Tiff", dir2+imageTitle);

    close();
}
```