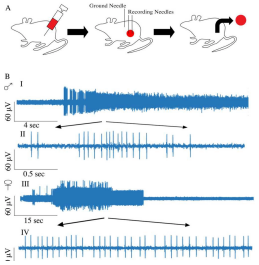
Jul 23, 2024

Neural recordings of spontaneously metastasizing melanomas and melanomas with low metastatic potential



DOI
dx.doi.org/10.17504/protocols.io.81wgbzn7ngpk/v1

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OPEN ACCESS

DOI: dx.doi.org/10.17504/protocols.io.81wgbzn7ngpk/v1

External link: https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0297281

Protocol Citation: Jay Shiralkar, Tiana Anthony, Grant A McCallum, Dominique Durand 2024. Neural recordings of spontaneously metastasizing melanomas and melanomas with low metastatic potential. **protocols.io** https://dx.doi.org/10.17504/protocols.io.81wgbzn7ngpk/v1

Manuscript citation:
Shiralkar J, Anthony T, McCallum GA, Durand DM (2024) Neural recordings can differentiate between spontaneously metastasizing melanomas and melanomas with low metastatic potential. PLoS ONE 19(2): e0297281. https://doi.org/10.1371/journal.pone.0297281

Protocol status: Working
We use this protocol and it's working

Created: June 18, 2024

Last Modified: July 23, 2024

Protocol Integer ID: 102033

Keywords: Melanoma, tumors, sympathetic nervous system, chronic recording, metastasis, autonomic nervous system, microneurography, murine, mouse, neuroelectric activity

Funders Acknowledgement:
CDMRP - BCRP
Grant ID: W81XWH-18-1-0581

Abstract

Multiple studies report that melanomas are innervated tumors with sensory and sympathetic fibers where these neural fibers play crucial functional roles in tumor growth and metastasis with branch specificity. Yet there is no study which reports the direct neural recording and its pattern during in-vivo progression of the cancer. We performed daily neural recordings from male and female mice bearing orthotopic metastasizing-melanomas and melanomas with low metastatic poential, derived from B16-F10 and B16-F1 cancer cells, respectively. Further, to explore the origins of neural activity, 6-Hydroxidopamine mediated chemical sympathectomy was performed followed by daily microneurographic recordings. We also performed the daily bioluminescent imaging to track in vivo growth of primary tumors and distant metastasis to the cranial area. The protocols contained herein describe the methods, materials and equipment used to accomplish this research.

Materials

MATERIALS

A	B	C	D	E
Protocol	**Description**	Manufacturer	**Part Number**	RRID
A. Mouse Models				
	C57BL/6J mice	Jackson Laboratories		RRID:IMSR_JAX:000664
	B16-F10	ATCC	CRL-6475-LUC2	RRID:CVCL_A4CJ
	B16-F1	ATCC	CRL-6323-LUC2	RRID:CVCL_A4CK
	Dulbecco's Modified Eagle's Medium (DMEM)	ATCC	30-2002	
	10% Fetal Bovine Serum (FBS)	ATCC	30-2020	
	1% Penicillin-Streptomycin Solution	ATCC	30- 2300	
	1X Phosphate Buffered Saline (PBS) (diluted)	Teknova	P3195	
	4% Paraformaldehyde (PFA)	Thermo Fisher	J19943.K2	
B. Chemical Sympathectomy				
	6-hydroxydopamine hyrobromide (6-OHDA)	Sigma-Aldrich	H116	
C. Neural Recordings				
	Isoflurane	Covetrus	29405	
D. Bioluminescence				

	A	B	C	D	E
	Imaging				
		D-luciferin salt	Gold Biotechnology	LUCK-1G	
		1X Phosphate Buffered Saline (PBS) (diluted)	Teknova	P3195	
	E. Histology				
		1X Phosphate Buffered Saline (PBS) (diluted)	Teknova	P3195	
		4% Paraformaldehyde (PFA)	Thermo Fisher	J19943.K2	
		Sucrose	Sigma-Aldrich	S0389	
		Neurofilament antibody (NF)	Thermo Fisher	2F11	RRID:AB_560286
		Tyrosine Hydroxylase antibody (TH)	Sigma-Aldrich	AB152	RRID:AB_390204
		Antigen Unmasking Solution, Citrate-Based - pH 6.0	Vector Labs	H-3300-250	
		Peroxidazed 1 (Ready-to-Use)	BioCare Medical	PX968G	
		Rodent Block M	BioCare Medical	RBM961H	
		Mouse-on-Mouse HRP-Polymer	BioCare Medical	MM620G	
		Betazoid DAB	BioCare Medical	BDB2004H	
		CAT Hematoxylin (Ready-to-Use)	BioCare Medical	CATHE-L; Lot no. 12513B	
		Rodent Decloaker, 10X, pH 6.6	BioCare Medical	RD913M / Lot no. 101608	
		Peroxidazed 1, Ready-to-Use	BioCare Medical	PX968M / Lot no. no070109-1	
		Rodent Block M: (Ready-to-Use)	BioCare Medical	RMB961/ Lot no. 091009-1 (Mouse tissues only)	

	A	B	C	D	E
		Rabbit-on-Rodent HRP Polymer: (Ready-to-Use)	BioCare Medical	RMR622H/ Lot no. 010214	
		Betazoid DAB	BioCare Medical	BDB2004L / Lot no. 092509	

EQUIPMENT

	A	B	C	D
	Protocol	**Description**	**Manufacturer**	**Part Number**
	A. Mouse Models			
		Anesthesia Machine	Parkland Scientific	SN. 1172
	B. Chemical Sympathectomy			
		Insulin syringes	Carepoint Vet	12-7903
	C. Neural Recordings			
		Micromanipulator	WPI	M3301R
		Microneurography needle (reference)	FHC	30084
		Microneurography needle (active)	FHC	30080
		Neural Amplifier (Neuro Amp EX)	AD Instruments	FE285
		Neuro Amp EX Head stage	AD Instruments	MLT185
		Signal Acquisition System	AD Instruments	PowerLab 8/35
		Signal Storage and Visualization	AD Instruments	LabChart v8.1.13

	A	B	C	D
		Software		
		Spike Sorting Software	Citation (2)	UltraMegaSort 2000
	D. Bioluminescence Imaging			
		BLI imaging system	Perkin-Elmer	In-Vivo Imaging System (IVIS)
		Insulin syringes	Carepoint Vet	12-7903
	E. Histology			
		20mL syringes	Nipro	JD+20L

B16-F10-Luc2 & B16-F1-Luc2 Cell Culturing Protocol

1 Store the vials containing cells in liquid nitrogen vapor until they are ready for use.

2 Thaw in a 37°C water bath, then decontaminate by dipping in or spraying with 70% ethanol. All of the operations from this point on will be carried out under strict aseptic conditions.

3 Add vial contents to a complete culture medium, and then centrifuge at approximately 125 x *g* for 5 to 7 minutes.

4 Add the cell pellet to the recommended complete medium. Medium: DMEM, 10% FBS, 1% Penicillin-Streptomycin

5 Incubate the culture at 37°C in a suitable incubator at proper CO_2 exposure: 5% CO_2

6 Renew medium every 2- 3 days: remove and discard old medium.

7 Rinse cell layer with 1X PBS

8 Add 2.0 to 3.0 mL of Trypsin solution to 75cm^3 flask and observe cells under an inverted microscope until the cell layer is dispersed (usually within 15 minutes). Corning ® T-75 flasks (catalog #430641) are recommended for subculturing this product.

9 Add 6.0 to 8.0 mL of complete growth medium and aspirate cells by gently pipetting

10 Add appropriate aliquots of the cell suspension to new culture vessels. Cultures can be established between 2 x 10^4 and 4 x 10^4 viable cells/cm^2

11 Incubate cultures at 37°C and 5% CO_2

12 Passage cells at least twice after thawing and before inoculation, but no more than 30 times total per cell
line.

13 Under isoflurane anesthesia (2%, 1L/min), each mouse underwent a subcutaneous injection with 5 x 10^5 cells to the flank area.

Chemical sympathectomy

14 Chemical sympathectomy solution was prepared by dissolving 6-hydroxydopamine (6-OHDA, 100 mg/kg body weight) in sterile saline along with 0.01% of ascorbate as a stabilizer. 2 mL of buffer per 5 mg vial provides a solution containing 10 mM 6-hydroxydopamine and 0.01% (w/v) ascorbic acid.

15 On the fourth (Day = -4) and second day (Day = -2) before cancer cell inoculation (Day = 0), the drug was injected intraperitoneally (IP) using an insulin syringe.

16 After these two initial injections, the drug was administered IP every five days to maintain the sustained effect of denervation. (i.e. Day = +5, +10, +15, etc)

Microneurography and neural recordings

17 Neural recordings were performed for thirty minutes for each day under 2% isoflurane in 1 L/min oxygen, for two weeks starting from day 6 post inoculation of cancer cells.

18 Microneurography needles were inserted into the primary tumor mass and a ground/reference needle was inserted on the contralateral side.

19 By using a micromanipulator, the needles were inserted at the same depth (5 mm) and same distance apart (4 mm), daily, in order to record from the same location. The two recording needles were fixed to a holder and were maintained at a 4 mm separation distance. The holder was mounted on a 1µm, x-y-z resolution micromanipulator system, so that the daily insertion depth could be repeated with certainty.

20 The neural data were sampled at 20 kHz and filtered from 500 Hz to 1200 Hz using a 7th order, zero-phase, digital bandpass filter.
ADInstruments amplifier (FE285) was used for all the recordings. Recorded neural data were displayed and stored in Lab Chart files.

21 Lab Chart files were exported into MATLAB (RRID:SCR_001622) compatible files.

22 The results were quantified in terms of number of spikes per file using UltraMegaSort2000 (UMS2000) spike sorting program (2) and neural spikes were plotted for ten minutes.

23 The averaged spike count per 10 minutes, was plotted as a function of days post inoculation of cancer cells.

Bioluminescence imaging

24 Images were acquired using Perkin-Elmer's In-Vivo Imaging System (IVIS) Spectrum at 60s exposure.

25 Under isoflurane anesthesia (2% at 1L/min), 200 µL of D-luciferin solution (12.5 mg/ml of D-luciferin in sterile phosphate buffer solution) was injected IP using an insulin syringe ten minutes before acquiring the images.

26 Total flux was quantified from the primary tumor region in order to quantify the in-vivo growth, as well as from the cranial region to quantify the growth of secondary metastatic foci.

27 Same sized regions of interest (ROIs) were used for the images with the same Field of View (FOVs) for a particular day of imaging.

Histology

28 At day 10 post-inoculation, under isoflurane anesthesia (2%, 1L/min) the mice were perfused via intracardiac perfusion first using 20 mL of 1X Phosphate Buffered Saline (PBS) solution followed by 20 mL of 4% Paraformaldehyde (PFA) solution.

29 The excised tumor tissues were then transferred to paraformaldehyde and refrigerated at 4°C for 24 to 48 hours.

30 After fixing in paraformaldehyde, the samples were immersed in a 15% sucrose solution with 1X PBS for 24 hours at 4°C.

31 Then, they were moved to a 30% sucrose 1X PBS solution and kept at 4°C until processing

32 The samples were formalin-fixed and paraffin-embedded, and 5µm thick slices were subjected to immunohistochemistry chromogenic detection using neurofilament primary antibody (NF, RRID: Thermo Fisher 2F11; TH, RRID: Sigma-Aldrich AB152) to detect the presence of nerve fibers and autonomic nerves, respectively.

*Tissues were fixed in 10% NBF , were embedded in paraffin, sectioned and mounted on slides , and then IHC stained.

	A	B	C	D	E	F	G	H	I
	Name	RRID	Source or reference	Catalog number	Antibody type	Target	Raised in	Clonality	Dilution used
	NEFL Monoclonal Antibody (2F11)	RRID:AB_560286	Thermo Fisher Scientific	MA1-06803	primary	NEFL	mouse	monoclonal	1:200
	Anti-Tyrosine Hydroxylase Antibody	RRID:AB_390204	Millipore	AB152	primary	Tyrosine Hydroxylase	rabbit	polyclonal	1:500

33 To obtain a comprehensive pathological understanding of the tumor microenvironment, slices were obtained from various tumor areas, ranging from about 100 μm.

34 **The neurofilament immunohistochemical (IHC) staining process was conducted as follows:**

34.1 Neurofilament Protocol:
Involved in the maintenance of neuronal caliber, neurofilaments are the intermediate filament proteins found specifically in neurons, and are composed predominantly of three major proteins called NF-L, NF-M and NF-H. Like most other intermediate filament proteins (IFPs), the expression of the different neuronal IFPs is both tissue-specific and developmentally regulated. NF-L is the light or low molecular weight microfilament subunit and runs on SDS-PAGE gels at approximately 70 kDa. Neurofilament are the 10nm or intermediate filament proteins found specifically in neurons, and are composed predominantly of three major proteins called NF-L, NF-M and NF-H. NF-H is the heavy or high molecular weight microfilament subunit and runs on SDS-PAGE gels in the range 180-220 kDa, with some variation in different species.

34.2 Bake slides at 60ºC for 75 min. (let cool for 20 min. after time elapses)

34.3 Deparaffinize slides in xylene, 2X for 7 min. each time

34.4 Rehydrate slides in graded ethanols:
a. 100% ethanol, 2X for 2 min. each time
b. 95% ethanol, 2X for 2 min. each time

c. 70% ethanol for 2 min.

34.5 Rinse slides in dH_2O. for 2 min. or use squirt bottle

34.6 ***[10X] Antigen Unmasking Solution/ Citric Acid Based- pH 6.0/ Vector Labs/ Cat#: H-3300***

Antigen retrieval:

a. Place slides in 250 mL [1X] Antigen Unmasking Buffer
b. Incubate in pressure cooker at 120 °C for 30 seconds
c. Remove slides and let cool on bench top for 20 min.

34.7 Rinse slides in dH_2O for 2 min.

34.8 ***Peroxidazed 1: (Ready-to-Use) / BioCare Medical / Cat#: PX968G***

Block endogenous peroxidase activity:

a. Place Slides in Peroxidazed 1 for 8 min.

34.9 Rinse slides in dH2O for 2 min.

34.10 ***Rodent Block M: (Ready-to-Use) / BioCare Medical/ Cat#: RBM961H /***

Block endogenous mouse IgG:

a. Place slides in Rodent Block M for 30 min.

34.11 Rinse slides in TBST

34.12 ***Mouse anti-Neurofilament (NEFL):Mouse Monoclonal Antibody IgG_1 /Invitrogen/ Cat#:MA1-06803/ Clone: 2F11/***

Apply primary antibody

a. [1:200] = X µL Neurofilament in X µL Diluent
b. Incubate slides at room temp. for one hour.

34.13 Rinse slides in TBST

34.14 ***Mouse-on-Mouse HRP-Polymer: (Ready-to-Use) / Biocare Medical /Cat#: MM620G***

Apply Mouse on Mouse HRP-Polymer for 30 min.

34.15 Rinse slides in TBST

34.16 ***Betazoid DAB: BioCare Medical / Cat#: BDB2004H***

Apply chromogen:

a. 1 drop DAB in 1 mL dH_2O
b. Incubate in Betazoid DAB for 5 min.

34.17 Rinse slides in dH_2O

34.18 ***CAT Hematoxylin: (Ready-to-Use) / BioCare Medical / Cat#: CATHE-L / Lot no. 12513B***

Counterstain with CAT Hematoxylin for1 min.

34.19 Rinse in running tap water for 30 seconds

34.20 Rinse in dH2O 3X for 1 min. each time

34.21 Blue the nuclei with ammonia water by dipping ten times

34.22 Rinse in running tap water for 30 seconds

34.23 Coverslip with resinous medium

34.24 Number of events per square millimeter of tissue slices were calculated in order to quantify and compare the staining metrics.

35 **The tyrosine hydroxylase (TH) immunohistochemical (IHC) staining process was conducted as follows:**

35.1 Tyrosine Hydroxylase plays an important role in the physiology of adrenergic neurons. It is the first and rate-limiting enzyme involved in the biosynthesis of the catecholamines Dopamine and Norepinephrine from tyrosine. TH is, therefore, a useful marker for dopaminergic and noradrenergic neurons. The enzymatic activity of TH requires ferrous ions as cofactors and is

believed to be regulated by phosphorylation. At least four isoforms of human TH have been identified which result from alternative splicing.

35.2 Bake slides at 60ºC for 75 min.

35.3 Deparaffinize slides in xylene, 2X for 7 min. each time

35.4 Rehydrate slides in graded ethanols:

a. 100% ethanol, 2X for 2 min. each time
b. 95% ethanol, 2X for 2 min. each time
c. 70% ethanol for 2 min.

35.5 Rinse slides in dH_2O

35.6 ***X Rodent Decloaker (pH 6.6): Biocare Medical / Cat#:RD913M / Lot no. 101608 / (To be diluted to 1 X)***

Antigen retrieval:

a. Place slides in 250 mL 1X Rodent Buffer
b. Incubate in Steamer at ~98°C for 20 minutes
c. Remove slides and let cool on bench top for 20 min.

35.7 Rinse slides in dH_2O

35.8 ***Peroxidazed 1: (Ready-to-Use / BioCare Medical / Cat#: PX968M / Lot no. no070109-1***

Block endogenous peroxidase activity:

a. Place Slides in Peroxidazed 1 for 8 min.

35.9 Rinse slides in dH_2O

35.10 ***Rodent Block M: (Ready-to-Use)/ BioCare Medical /Cat#: RBM961 / Lot no. 091009-1 (MOUSE TISSUES ONLY***

Block endogenous mouse IgG and non-specific background staining:

a. Place select slides in Rodent Block M for 20 min.

35.11 Rinse slides in TBST

35.12 ***Rabbit Polyclonal Tyrosine Hydroxylase Antibody: Millipore/ Cat#: AB152/ Lot no. JC1682878***

Apply primary antibodies

a. [1:500] = X µL of TH stock in X µL Diluent
b. Incubate slides overnight at 4° C.

35.13 Rinse slides in TBST

35.14 ***Rabbit-on-Rodent HRP Polymer: (Ready-to-Use)/ BioCare Medical/ Cat#:RMR622H/ Lot no. 010214***

Apply Rabbit Polymer HRP for 30 min.

35.15 **Rinse** slides in TBST

35.16 ***Betazoid DAB: BioCare Medical / Cat#: BDB2004L / Lot no. 092509***

Apply chromogen:

a. Incubate in Betazoid DAB for 5 min.
b. 1 drop DAB in 1.0 mL dH_2O

35.17 Rinse slides in dH_2O

35.18 ***CAT Hematoxylin: (Ready-to-Use) / BioCare Medical / Cat#: CATHE-L / Lot no. 12513B***

Counterstain with CAT Hematoxylin for 30 secs.

35.19 Rinse in running tap water for 30 seconds

35.20 Rinse in dH_2O 3X for 1 min. each time

35.21 Blue the nuclei with TBST wash buffer

35.22 Rinse in running tap water for 30 seconds

35.23 Air dry overnight or dehydrate to Xylene

35.24 Coverslip with resinous medium

35.25 Number of events per square millimeter of tissue slices were calculated in order to quantify and compare the staining metrics.

Protocol references

1. Fazakas C, Wilhelm I, Nagyoszi P, Farkas AE, Haskó J, Molnár J, et al. Transmigration of melanoma cells through the blood-brain barrier: role of endothelial tight junctions and melanoma-released serine proteases. PloS One. 2011;6(6):e20758.
2. Hill DN, Mehta SB, Kleinfeld D. Quality Metrics to Accompany Spike Sorting of Extracellular Signals. J Neurosci. 2011 Jun 15;31(24):8699–705.