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14. ABSTRACT

Chronic neural recordings within the 4T1 mouse tumor model have identified two distinct days where there is high neural activity during the growth of the tumor. The first day occurs approximately 4 days before metastasis and the second day occurs primarily on the day of metastasis.

Furthermore, preliminary data in vagotomized mice with 4T1 tumors (n=2), all recorded neural activity within the tumor is eliminated and metastasis occurs approximately six days earlier compared to mice with intact vagus nerves. Additional experiments are ongoing to substantiate this result.

15. SUBJECT TERMS

Breast cancer, metastasis, autonomic nervous system, vagus nerve

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1. **INTRODUCTION:** The goal of this project is to determine the role autonomic nervous system activity, recorded within solid tumors, has as an indicator of breast cancer tumor growth and metastasis. Furthermore, evidence suggests that vagus nerve stimulation could help prevent breast cancer recurrence and metastasis. If so, it could lead to a more effective and less toxic treatment regimen compared to existing treatment options. The project is focused on the interaction between the autonomic nervous system and the breast tumor.

2. **KEYWORDS:** Breast tumor cancer autonomic nervous system metastasis vagus nerve stimulation recurrence chronic neural recording

3. **ACCOMPLISHMENTS:**

   - **What were the major goals of the project?**

     - AIM#1: Create an indicator of breast cancer tumor growth and metastasis by recording neural activity within a breast tumor.
     - AIM#2: Develop a more effective and less toxic treatment regimen compared to existing treatment options by vagus nerve stimulation (VNS)
     - IACUC and ACURO approval for animal protocol.
     - Create additional recording boards for simultaneous, multi-subject neural recordings.
     - Configure and validate multi-channel neural stimulation system.

   - **What was accomplished under these goals?**

     - Both the multi-channel neural recording and stimulation systems were completed and functionally verified to enable the necessary experiments for the project.
     - Proficient surgical skills and techniques have been developed by multiple members of the research team to perform the implants and procedures required for the project.
     - Performing chronic recordings in normal 4T1 (triple-negative) tumor bearing mice resulted in the identification of two distinct days during tumor development when a significant increase in neural activity was detected within the tumor (n=10). These two days were consistently around day 16 and day 20 (post-inoculation). The second peak day also was significantly correlated with the day of metastasis determined from BLI imaging.
     - **Figure 1** (Appendix) gives a high-level overview of the chronic tumor recording setup as two electrodes are implanted within the breast tumor and two additional electrodes are implanted on the contralateral side as used as a control. The neural activity within the tumor and control are amplified, filtered and digitized and stored to a laptop for further processing. Representative waveforms for the tumor and control signals are also displayed.
     - As seen in **Figure 2** (Appendix), the electrode impedance (n=40) for all mice over the entire tumor recording period was stable and did not change indicating a stable recording interface.
     - **Figure 3** (Appendix): **(A)** shows a representative mouse and the recorded neural spike counts from days 12-25 post-inoculation in which the two days of high spike peak activity can be seen for the 4T1 model. **(B)** shows BLI imaging for a mouse with 4T1 cancer as the tumor grows and metastasis occurs. **(C)** Indicates there is a significant difference in the time between the first peak in activity and the second peak and also for metastasis, however, there is no significant time difference between the second peak in activity compared to the day of metastasis. **(D)** shows the significant amount of neural activity within the tumor on the peak activity days compared to the recorded control side activity.

- **Figure 4** (Appendix): Histology of a day-7 post-inoculation 4T1 tumor showing the presence of neurofilments (red), DAPI (blue) and finally two composite images showing a large section of the tumor and another higher magnification image showing more detail of the neural structures.

- **What opportunities for training and professional development has the project provided?**
  - Nothing to Report
- **How were the results disseminated to communities of interest?**
  - Co-I, Dr. Jennifer Yu, discussed the tumor recording results with a colleague (Dr. Brian Gastman) at the Cleveland Clinic Foundation. Dr. Gastman is a world-renowned plastic surgeon and he is very interested to understand if the same techniques can be applied to melanomas since it has been widely known that nerve fibers are present in this cancer type.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - Now that all the technical issues have been resolved (multi-channel recording and stimulation systems built and optimized, surgical techniques perfected, and staff built up) we are planning on experimenting on eight (8) mice per month and each cancer cell type (i.e.. 4T1 and MMTV-neuT) will be alternated every month. This will allow us to gather data on all of the experimental groups in Aim #1 and begin to understand the autonomic nerve effects not only between the different groups, but also between different cancer types.
  - A second manuscript will be written and submitted with all the Aim #1 results.

4. **IMPACT:** *Describe distinctive contributions, major accomplishments, innovations, successes, or any change in practice or behavior that has come about as a result of the project relative to:*

- **What was the impact on the development of the principal discipline(s) of the project?**
  - Nothing to Report
- **What was the impact on other disciplines?**
  - Nothing to Report
- **What was the impact on technology transfer?**
  - There exists a real potential to create a medical device to perform a real-time biopsy using the neural recording techniques within the cancer mass. Early adopters would be dermatologists since cancer-types, like melanomas, where access to the cancerous tissue is easily accessible.
- **What was the impact on society beyond science and technology?**
  - Nothing to Report

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**
  - Nothing to Report
- **Actual or anticipated problems or delays and actions or plans to resolve them**
  - The original plan to purchase MMTV-neuT mice and wait until they spontaneously developed tumors was unrealistic for the grant timeline since these tumors can take up to 20 weeks to develop. So, we purchased cells from the identical tumor line from ATCC and will begin to directly inoculate them identical to what is done with the 4T1 cell line. These MMTV-neuT cells were also transfected with Lucifrase so BLI imaging could also be performed.

- BLI imaging proved to be time consuming since we have to image the mice daily in a core facility across campus which involves transporting the mice back and forth. In lieu of this inconvenience, we've recruited a CWRU undergraduate and the department Veterinarian Technician to help perform the BLI imaging.

- **Changes that had a significant impact on expenditures**

  - Cost reduction: As indicated above, the cost of MMTV-neuT mice experiments was reduced by purchasing just the cancer cells and inoculating instead of purchasing the entire transgenic mouse model. This cost reduction is offset by the higher than anticipated BLI imaging costs.

  - Cost reduction: A new connector was developed for the vagus nerve stimulation experiments since only two leads are required to deliver the stimulus waveform. Small permanent magnets are embedded in a 3D-printed housing. This design provides a lighter connector for the chronic experiments that is also easier to attach and detach from the animal. This design provides a 75% reduction in cost over standard off-the-shelf connector components. See **Appendix Figure 5** below for details.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**

  - Significant changes in use or care of human subjects. **(Not Applicable)**

  - Significant changes in use or care of vertebrate animals. **(None)**

  - Significant changes in use of biohazards and/or select agents. **(None)**

6. **PRODUCTS:**

- **Publications, conference papers, and presentations**

  - **Journal publications.**

    - Grant A. McCallum, Jay Shiralkar, Gil Covarrubias, Efstathios Karathanasis, Jennifer Yu, Dominique M. Durand. *Recording chronic neural activity within breast tumors.* In manuscript preparation.

- **Other Products**

  - Data: A significant number of raw neural recording data from within the breast tumor has been collected in addition to corresponding BLI images on the same day. This database will continue to grow as the project continues and more experimental groups are completed.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**

Name:	Dominique Durand
Project Role:	PI
Researcher Identifier (e.g. eRA Commons):	DDURAND
Nearest person month worked:	*10*
Contribution to Project:	Overseeing all aspects of the project.
Funding Support:	No change from proposal funding documents

Name:	Jennifer Yu
Project Role:	Co-I
Researcher Identifier (e.g. eRA Commons):	JENNYU
Nearest person month worked:	*5*
Contribution to Project:	Coordinating all MMTV cell preparation activities.
Funding Support:	No change from proposal funding documents

Name:	Efstathios Karathanasis
Project Role:	Co-I
Researcher Identifier (e.g. eRA Commons):	ekarathanasis
Nearest person month worked:	*5*
Contribution to Project:	Coordinating all 4T1 cell preparation activities.
Funding Support:	No change from proposal funding documents

Name:	Grant McCallum
Project Role:	Project Manager, Research Associate
Researcher Identifier (e.g. eRA Commons):	GMCCALLUM
Nearest person month worked:	*60*
Contribution to Project:	Dr. McCallum has performed work in the areas of program management, surgery, recordings, BLI imaging, vagus nerve stimulation.
Funding Support:	No change from proposal funding documents

Name:	Jay Shiralkar
Project Role:	Graduate Student
Researcher Identifier (e.g. eRA Commons):	N/A
Nearest person month worked:	*140*
Contribution to Project:	Mr. Shiralkar has performed work in the areas of surgery, recordings, BLI imaging and histology.
Funding Support:	Self-funded

Name:	Gil Covarrubias
Project Role:	Graduate Student
Researcher Identifier (e.g. eRA Commons):	N/A

Nearest person month worked:	*10*
Contribution to Project:	Mr. Covarrubias has performed work in 4T1 cell preparation and inoculations.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  - Nothing to report.
- **What other organizations were involved as partners?**
  - **Organization Name: Cleveland Clinic Foundation**
  - **Location of Organization:** Cleveland, Ohio, USA
  - **Partner's contribution to the project**
  - **Facilities**: Lab of Dr. Jennifer Yu, MD, PhD to perform MMTV-neuT cell preparation and transfect the MMTV-neuT cells with luciferase to enable BLI imaging of mice.
  - **Collaboration**: Coordinated with Dr. Yu's lab member to drop-off and pick-up MMTV-neuT cells for inoculation performed at CWRU.

8. **APPENDICES:**

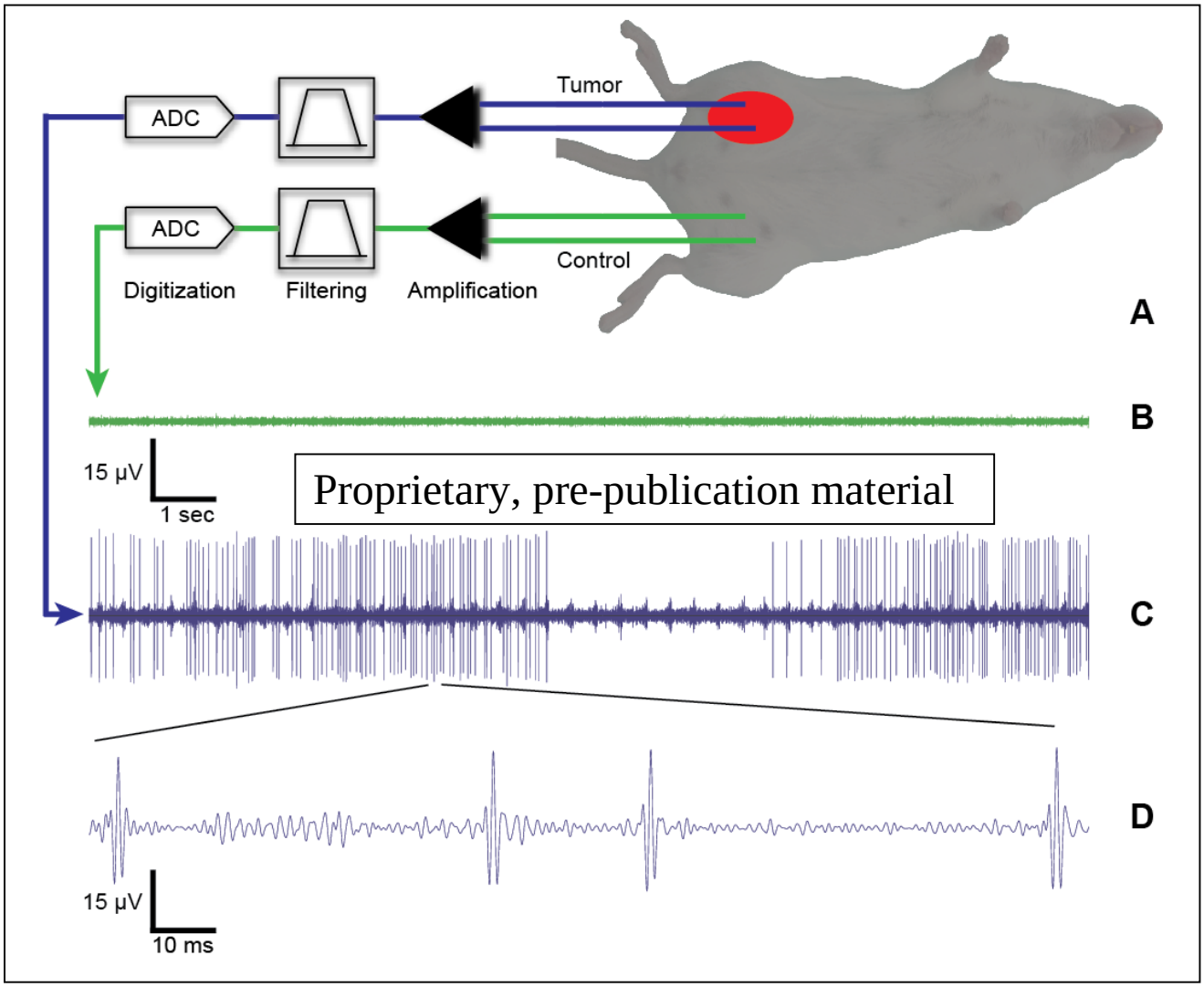

Figure 1: Chronic recording setup for neural activity recording within breast tumors in mice. (A) Two differential recording channels are used to simultaneously record from the tumor and the contralateral side as a control. (B) Control side waveform showing no neural activity. (C) Tumor activity waveform with clearly identifiable neural spike activity. (D) Magnified section of tumor activity showing the neural spike activity.

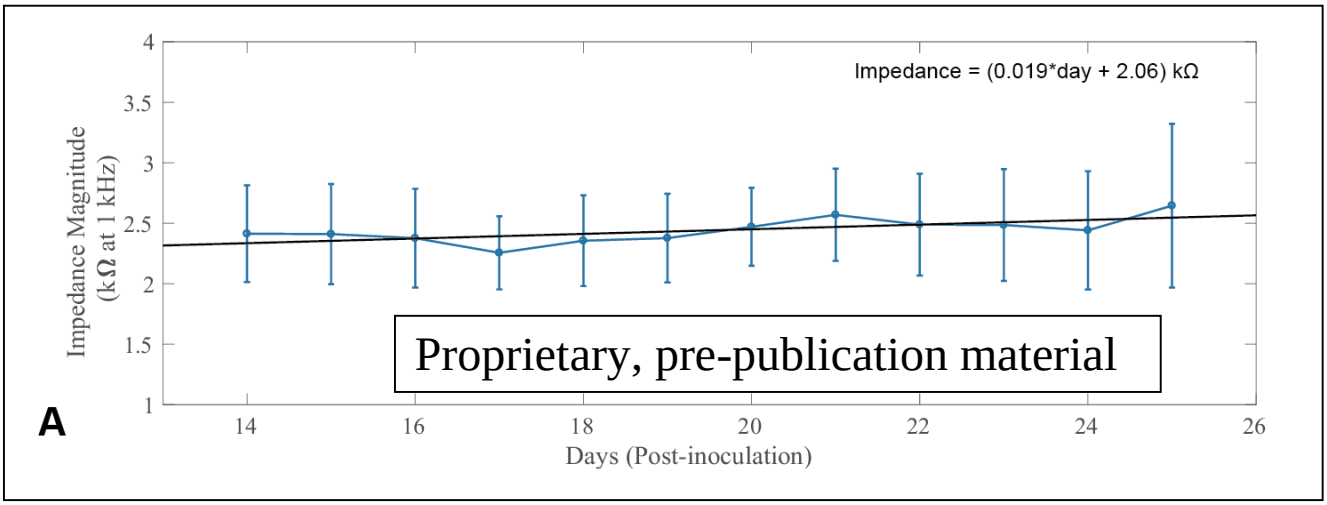

Figure 2: Electrode impedance over time. Combined impedance data of both the tumor and control electrodes (n=40). The stable electrode impedance over the 11-day implant period indicates a stable neural recording interface.

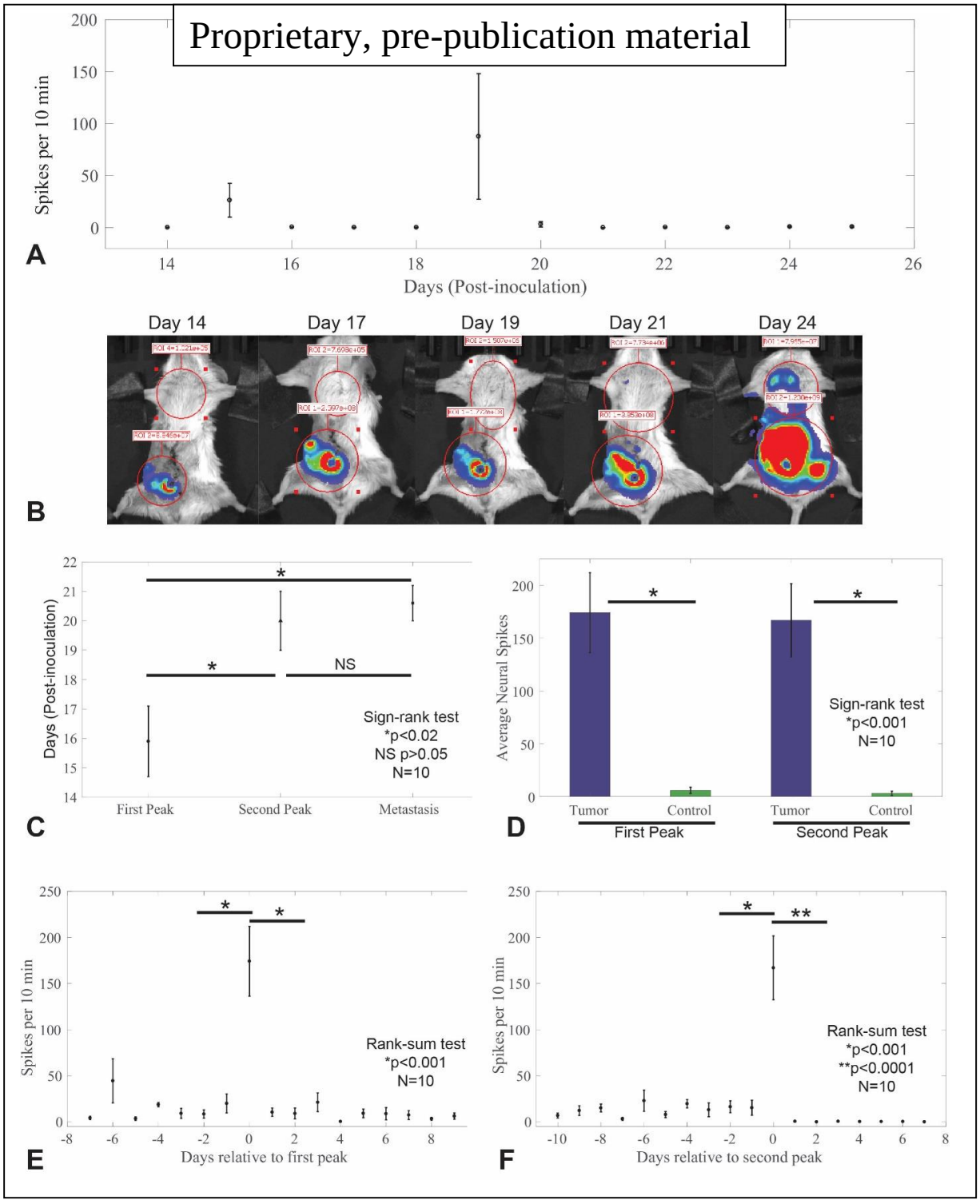

Figure 3: Summary of 4T1 control group tumor activity. (A) Representative tumor activity over an 11-day period showing the two peak days (Day 15 & 19) of neural activity within the tumor. (B) Representative BLI imaging results of a mouse showing the tumor growth and metastasis (Day 21). (C) Days post-inoculation when first and second peak activity and metastasis occurred. (D) Significant spike activity comparing the tumor to the control signal during both the first and second peak days. (E & F) The peak activity days for all

10 mice were lined up on the peak day (i.e. Day 0). Significant differences between activity both before and after both peak days was found.

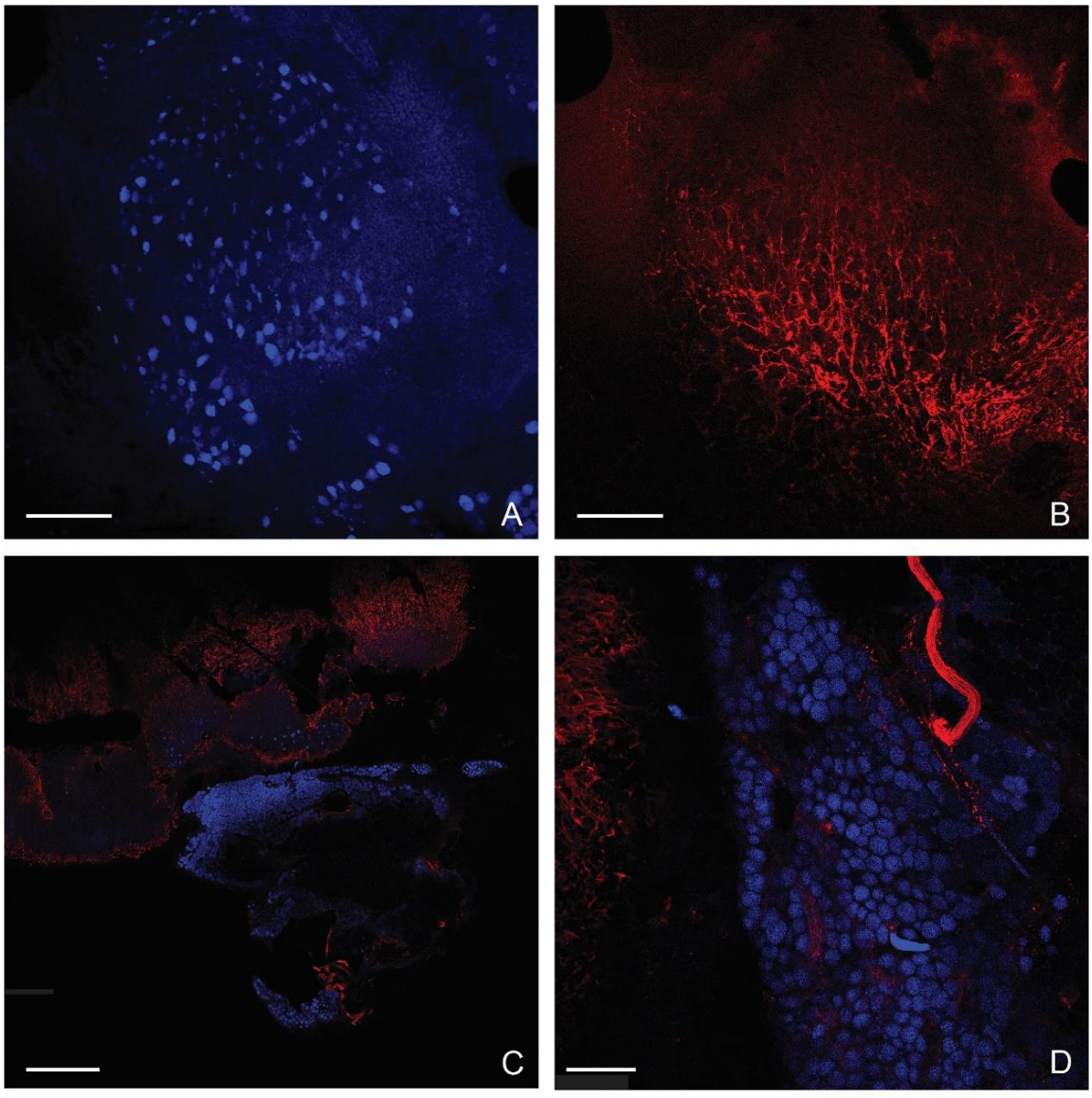

Figure 4: Histological analysis of the 4T1 tumor and presence of neurofilaments. (A) DAPI stain [scale bar = 250 µm], (B) Neurofilament (NF) stain [scale bar = 250 µm], (C) Large view of composite DAPI and NF [scale bar = 1 mm], (D) Higher magnification image of composite [scale bar = 200 um].

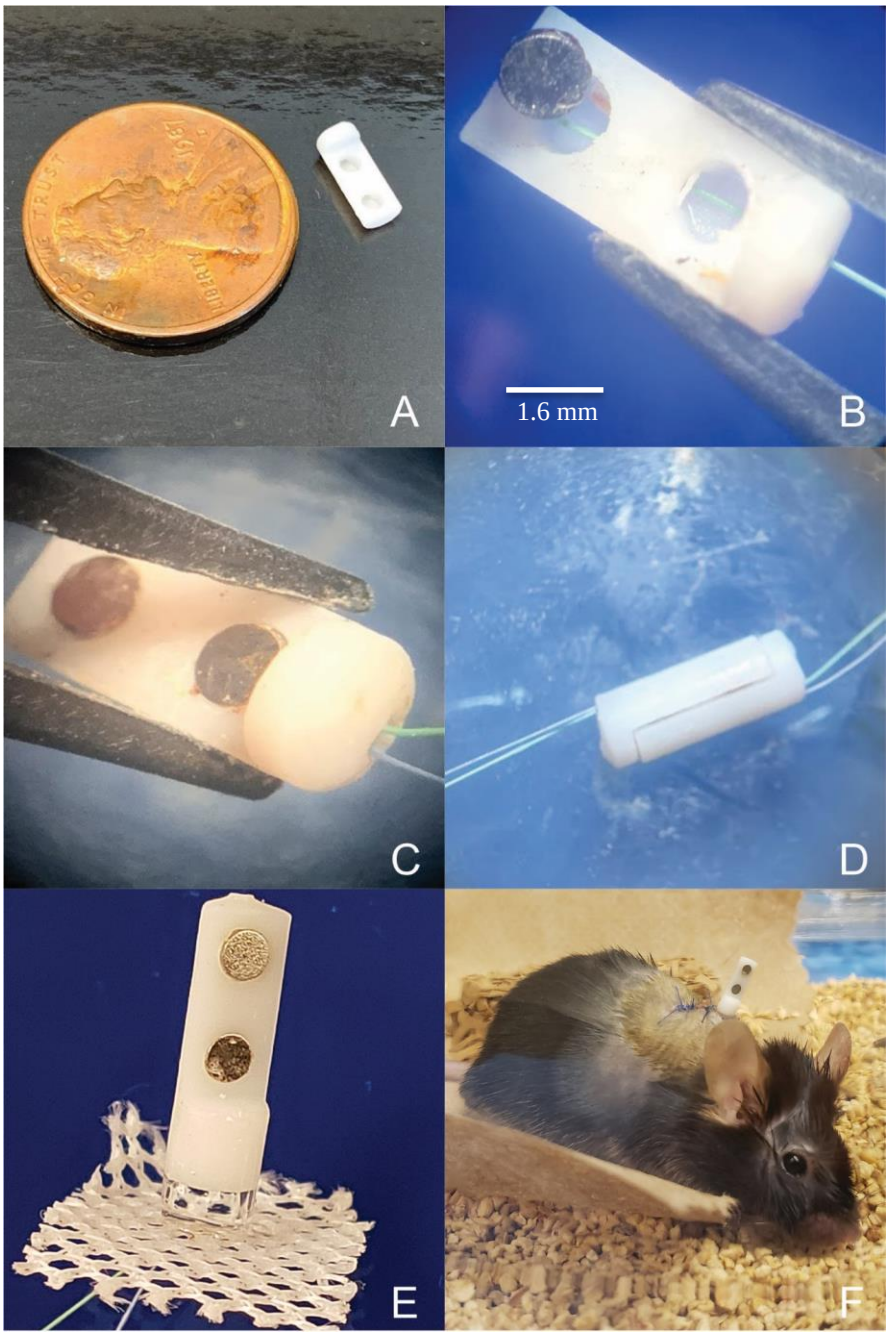

Figure 5: Magnetic connector for chronic vagus nerve stimulation (AIM#2). (A) 3D-printed housing with holes to hold permanent magnets and a channel to route the wire connections. (B) Wires are soldered to one side of the magnet and the wires are threaded through the channel. (C) Magnets are superglued in position within the housing. (D) Two identical connectors are mated together. One set of wires will go to the implanted cuff electrode around the vagus nerve the other set of wires will be connected to a commutator which is connected to the external neural stimulation system. (E) Decron mesh is fixed to the connector using UV curable epoxy. During implantation, sutures are used to secure the Decron mesh to the mouse's muscle fascia. (F) Implanted connector on the dorsal side of a mouse.