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## Induction of a Brain Metastasis Mouse Model Using Internal Carotid Artery Injection Route. --Manuscript Draft--

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<b>Corresponding Author:</b>	Amr Morsi NYU Langone Medical Center New York, NY UNITED STATES
<b>Corresponding Author Secondary Information:</b>	
<b>Corresponding Author E-Mail:</b>	amourias@hotmail.com
<b>Corresponding Author's Institution:</b>	NYU Langone Medical Center
<b>Corresponding Author's Secondary Institution:</b>	
<b>First Author:</b>	Amr Morsi
<b>First Author Secondary Information:</b>	
<b>Other Authors:</b>	Evelyn B. Voura
	John G. Golfinos
	Youssef Z. Wadghiri
<b>Order of Authors Secondary Information:</b>	
<b>Abstract:</b>	In experimental models of human diseases, intra-carotid cannulation has been instrumental to allow for the injection of cells and other varied substances into the blood stream for their consequent delivery to the brain. Since the carotid artery is a main vessel supplying the brain, this delivery route is particularly important for agents having short blood half-lives, those that would end up in the general periphery, or those that would be rapidly excreted by either the kidneys or the reticuloendothelial system prior to their entry to the organ of interest. Herein we present the steps required for infusing cells into the mouse internal carotid artery, as a method for their direct delivery to the central nervous system. We used this technique to induce a mouse model of brain metastasis. The details of the microsurgical technique are described using a widely studied inbred strain of laboratory mice - C57 Black 6 or C57Bl/6. Although we chose the B16F10 murine melanoma cell line, this technique is easily amenable to other cells of interest.
<b>Author Comments:</b>	Thank you for re-considering our manuscript entitled "Induction of a Brain Metastasis Mouse Model Using an Internal Carotid Artery Injection Route." for publication in Journal of Visualized Experiments. Please find enclosed a revised version of the manuscript. We would like to thank reviewers for their detailed and useful comments and believe that they have helped strengthen this paper. We have addressed and incorporated all their comments. We hope that this revised manuscript is now suitable for publication in Journal of Visualized Experiments.
<b>Additional Information:</b>	
<b>Question</b>	<b>Response</b>



**Bernard & Irene Schwartz Center for Biomedical Imaging**  
**Department of Radiology**  
**NEW YORK UNIVERSITY SCHOOL OF MEDICINE**



Youssef Zaim Wadghiri, Ph.D.  
Assistant Professor of Radiology  
Director of Imaging Shared Resource

660 First Avenue, Room 444 New York, NY 10016  
Phone: 212-263-3336  
Facsimile: 212-263-7541  
E-mail: wadghiri@med.nyu.edu

New York, May 25<sup>th</sup>,  
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Cambridge, MA 02139 USA

Dear Joni Williams,

We are submitting our manuscript entitled, "Induction of a Brain Metastasis Mouse Model Using Internal Carotid Artery Injection Route" for your consideration. Our study demonstrates, in depth, the steps of internal carotid artery injection as a method for the direct delivery of cancer cells to the central nervous system (CNS) in order to induce brain metastasis. We believe this work would be of interest to the broad audience of *The Journal of Visualized Experiments* as it explains in detail the microsurgical technique necessary for a successful induction of brain metastases in mice. Preclinical models of brain metastasis, such as ours, are fundamental to the study of brain metastasis and are critical to evaluate the efficacy of new therapeutic regimens. Applying our microsurgical protocol, we successfully established an experimental model of brain metastasis using the B16F10 melanoma cell line - the most widely used experimental melanoma cell line - and the C57 Black 6 (C57BL/6) mouse strain.

By signing this letter, we certify that the content of the manuscript is original and that it has not been published or accepted for publication, either in whole or in part, in any form (other than as an abstract). The manuscript has been formatted as a short communication, and it contains 1,926 words (abstract, introduction, protocol, results and discussion), 3 figures, and 1 Excel Spreadsheet-Table of Materials/Equipment. We also guarantee that no part of the manuscript is currently under consideration for publication elsewhere.

Thank you for your time and consideration.

**Conflict of interest:** The authors declare no conflict of interest.

Sincerely,

Youssef Zaim Wadghiri, Ph.D.

Assistant Professor  
Department of Radiology,  
New York University School of Medicine



**TITLE:** Induction of a Brain Metastasis Mouse Model Using an Internal Carotid Artery Injection Route.

**AUTHORS:**

Morsi, Amr, M.D.

Departments of Neurosurgery & Radiology, NYU Langone Medical Center  
660 First Ave 4th Floor Room 444, New York, NY 10016  
Tel: 001 (917) 696 3297; Fax: 001 (212) 263-2950  
Email: [amourias@hotmail.com](mailto:amourias@hotmail.com)

Voura, Evelyn B., Ph.D.

Department of Biology, Dominican College  
470 Western Highway, Orangeburg, NY 10962 &  
Departments of Neurosurgery & Radiology, NYU Langone Medical Center  
660 First Ave 4th Floor Room 444, New York, NY 10016  
Tel: 001 (845) 848-6011; Fax: 001 (212) 263-2950  
Email: [evelyn.voura@dc.edu](mailto:evelyn.voura@dc.edu)

Golfinos, John G., M.D.

Department of Neurosurgery, NYU Langone Medical Center  
530 First Ave Suite 8R, New York, NY 10016  
Tel: 001 (212) 263-2950; Fax: 001 (212) 263-2950  
Email: [john.golfinos@nyumc.org](mailto:john.golfinos@nyumc.org)

Wadghiri, Youssef Z., Ph.D.

Department of Radiology, NYU School of Medicine  
The Bernard & Irene Schwartz Center for Biomedical Imaging,  
660 First Ave 4th Floor Room 444, New York, NY 10016  
Tel: 001 (212) 263-3336; Fax: 001 (212) 263-7541  
Email: [wadghiri@med.nyu.edu](mailto:wadghiri@med.nyu.edu)

**CORRESPONDING AUTHOR:**

Morsi, Amr, M.D.

Departments of Neurosurgery & Radiology, NYU Langone Medical Center  
530 First Ave Suite 8R, New York, NY 10016  
Tel: 001 (917) 696 3297; Fax: 001 (212) 263-2950  
Email: [amourias@hotmail.com](mailto:amourias@hotmail.com) &

Wadghiri, Youssef Z., Ph.D.

Department of Radiology, NYU School of Medicine, NYU Langone Medical Center  
The Bernard & Irene Schwartz Center for Biomedical Imaging,  
660 First Ave 4th Floor Room 444, New York, NY 10016  
Tel: 001 (212) 263-3336; Fax: 001 (212) 263-7541  
Email: [wadghiri@med.nyu.edu](mailto:wadghiri@med.nyu.edu)

**KEYWORDS:** Internal Carotid Artery Injection, Brain Metastasis, Mouse Model.

**SHORT ABSTRACT:** *(10 words minimum, 50 words maximum)*

This protocol provides a step-by-step procedure for the successful delivery of cells to the mouse brain following their injection into the internal carotid artery.

**LONG ABSTRACT:** *(150 words minimum, 300 words maximum)*

In experimental models of human diseases, intra-carotid cannulation has been instrumental to allow for the injection of cells and other varied substances into the blood stream for their consequent delivery to the brain. Since the carotid artery is a main vessel supplying the brain, this delivery route is particularly important for agents having short blood half-lives, those that would end up in the general periphery, or those that would be rapidly excreted by either the kidneys or the reticuloendothelial system prior to their entry to the organ of interest. Herein we present the steps required for infusing cells into the mouse internal carotid artery, as a method for their direct delivery to the central nervous system. We used this technique to induce a mouse model of brain metastasis. The details of the microsurgical technique are described using a widely studied inbred strain of laboratory mice - C57 Black 6 or C57Bl/6. Although we chose the B16F10 murine melanoma cell line, this technique is easily amenable to other cells of interest.

**INTRODUCTION:** *(150 words minimum, 1500 words maximum)*

Metastatic brain lesions represent neoplasms originating in extracranial tissue that spread secondarily to the brain via hematogenous routes. Metastasis to the brain occurs in 15-30% of cancer patients and the prognosis for these patients is dismal with an average of 4-6 months survival from diagnosis (1-4). Current treatments for brain metastases are determined by their number and location in the brain. Patients with a limited number of resectable brain metastases may undergo surgical resection or stereotactic radiosurgery, while those with more disseminated disease are usually treated with whole-brain radiation therapy or chemotherapy (3,5,6).

Preclinical studies of brain metastases depend greatly on experimental mouse models to mimic the human pathology (4,7,8). Such models have been primarily developed by the introduction of neoplastic cells directly into the arterial circulation through internal carotid artery injection. This technique simulates the hematogenous stage of malignant cell dissemination from a primary tumor to distant organs providing investigators with a means to study the arrest and extravasation of cancer cells. Moreover, this means of tumor induction produces a high incidence of brain metastases and a low frequency of visceral spread, thereby providing a great advantage as a method to study brain metastases (8).

In this protocol, we outline, in detail, the steps we used for the internal carotid artery injection of cancer cells to the central nervous system in order to induce brain metastasis. While the details of the microsurgical technique are described using C57 Black 6 (C57BL/6) mice and the B16F10 murine melanoma cell line, this method can easily be applied to other mouse strains and cell lines of interest. As an extension to our protocol, to evaluate the success of the surgery, the injected mice were subsequently scanned using three-dimensional MRI to detect brain metastases. This imaging approach also proved very useful to non-invasively evaluate the progress of tumorigenesis (9). As a final analysis, we also validated our MRI findings using conventional histological staining.



**PROTOCOL:**

The animals used in this study were maintained and cared for under the protocols approved by the Institutional Animal Care and Use Committee of New York University Langone Medical Center.

1. Begin with, as is generally prescribed, young mice (6 - 8 weeks of age) with a body weight ranging from 20 to 25 grams.
2. Sterilize instruments by autoclaving (minimum 121°C, 15 PSI, for 15 min.) and sanitize the surgery table and associated equipment using 70% ethanol.
3. During melanoma cell preparation, be sure that all trypsin is neutralized using serum and wash cells in PBS three times prior to counts. Prepare cell dilution in PBS to a concentration of  $1 \times 10^5$  cells/100 $\mu$ l. Keep cells on ice and swirl periodically to prevent the cells from clumping till the time of injection.
4. Place the mouse in an anesthetic induction chamber that is not much larger than the animal. Use a vaporizer to easily induce and maintain gaseous anesthesia for the extended time required for this procedure. Induce the mouse with an initial 4-5.0% isoflurane in air. Anesthesia will set in within 2 to 3 minutes.
5. Verify the depth of anesthesia by a toe pinch.
6. Wait for 2 minutes before continuing the procedure, even if the animal appears unresponsive.
7. Stage the mouse by inserting a nose cone to continue the delivery of the gas.
8. Avoid inhalation by using the appropriate gas scavenging equipment, as sustained exposure will cause operator drowsiness. The setup should be operated with an F/air canister with an interchangeable filter cartridge either under a hood, a device with suction/extraction or in a well-ventilated room.
9. Following the induction of anesthesia, reduce the level of isoflurane delivered to 1.5% and maintain this concentration for the rest of the protocol using a low volume air flow of approximately 0.5 to 1.0 liters per minute.
10. Place the mouse on the surgical stage in a supine position (belly side up or dorsal recumbence). Tape the fore paws palms up and the hind paws sole down to the stage. Extra caution should be taken when securing the mouse to the stage to ensure that the neck and spine are aligned in a straight line in such a manner to also prevent any deviation or rotation during the procedure.
11. Use a mouse rectal probe (1 mm diameter) to monitor body temperature, and maintain it at 35 to 37°C with a controllable heating pad throughout the surgical procedure.

12. Apply artificial tears or antibiotic ophthalmic ointment to both eyes to protect the corneas.
13. Remove the fur from the throat and left neck region beyond the prospective incision site by applying a generous amount of depilatory cream.
14. Clean off the depilatory agent and apply liberal amount of Betadine to disinfect the skin. Apply this agent by starting from the center of the surgical region, spiraling outward.
15. Rinse with sterile gauze pad containing 70% ethanol, moving the pad in a similar pattern as for the Betadine.
16. Repeat both 13 and 14 for a total of three cycles.
17. Make a midline incision at the neck extending laterally with scalpel blade #15 and a scalpel handle #3.
18. Position a dissection microscope over the staged mouse.
19. Using the microscope as an aid, begin by bluntly dissecting the surgical field and gently retracting the omohyoid and sternocleidomastoid muscles to expose the bifurcation of the left common carotid artery (CCA) into external carotid artery (ECA) and internal carotid artery (ICA). Great care should be exercised not to harm the vagus nerve.
20. Place a ligature using a 5-0 silk suture in the CCA.
21. Place a second ligature in the ECA.
22. Place a third ligature in ICA, proximal to the injection site and have it ready to be tied loosely over the cannula.
23. Gently pull the ICA suture laterally and apply a continuous traction, thereby producing a kink in the ICA.
24. At the location having the widest vessel diameter, nick the CCA with a pair of spring microscissors, just proximal to the bifurcation into ICA and ECA.
25. Insert the tip of a thinned out polyethylene PE-10 microbore tubing attached to a 1 ml luer lok syringe having a 27.5 Gauge needle containing the cell suspension, into the CCA through the arteriotomy.
26. Advance the cannula distally with a pair of forceps into the ICA to a point beyond the loosely tied ICA suture while slowly releasing tension on the suture to straighten the vessel.
27. Slowly inject 100 $\mu$ l cell suspension at a concentration of ( $1 \times 10^5$  cells/100 $\mu$ l) into the ICA

over 1 minute and pull out the cannula while tightening the third ligature.

28. Close the incision with 3-0 silk suture.

29. Turn off the isoflurane supply at the end of the surgical session, increase the air-flow to 1.5 to 2 liters per minute, and place the mouse in a 35°C nursing box to recover from anesthesia.

30. Apply a heat source to aid recovery. The animal will awaken and be alert in 3 to 5 minutes.

31. Once fully recovered (typically in approximately 10 minutes), return the mouse to the cage.

### **REPRESENTATIVE RESULTS:**

Schematic illustration of the surgical steps needed for injection of tumor cells into the internal carotid artery of C57Bl6 mouse as shown in Figure 1. The graph in figure 2 depicts the noticeable post-operative body weight loss usually observed in mice subjected to intra-carotid surgery. Successful introduction of tumor cells into the arterial circulation resulted in the development of melanoma brain metastatic lesions as shown by MRI and confirmed by histology, Figure 3.

### **FIGURE LEGENDS:**

**Figure 1: Internal Carotid Artery Cannulation.** (a) A representative simplified diagram of the arterial Circle of Willis of a mouse brain. CCA, common carotid artery; ECA, external carotid artery; ICA, internal carotid artery; PComA, posterior communicating artery; MCA, middle cerebral artery; ACA, anterior cerebral artery; AComA, anterior communicating artery; BA, basilar artery; PCA, posterior cerebral artery; PPA, pterygopalatine artery; ST, superior thyroid artery; OA, occipital artery. (b) A permanent ligature suture is placed on the left common carotid (CCA) and the left external carotid (ECA) arteries in preparation for left internal carotid artery (ICA) injection. (c) A third ligature is placed loosely over the ICA, proximal to the injection site and gently pulled laterally to minimize back-flow bleeding and provide hemostasis once the artery is nicked. (d) Arteriotomy is performed at the bifurcation of CCA. (e) A cannula is introduced through the CCA opening into the ICA and melanoma cell suspension is injected. (f) The cannula is pulled out while tightening the ligature.

### **Figure 2: Changes in mouse weight following internal carotid artery injections**

A representative graph of mean weight following melanoma cell injection ( $1 \times 10^5$  cells/100  $\mu$ l/mouse) into C57/Bl6 mice (n=10) using internal carotid artery injection surgery. The mice were monitored for weight changes on a daily basis post operatively.

**Figure 3: Peripheral metastatic brain lesion:** (a) A representative coronal T1-weighted MR image from an experimental B16F10/C57Bl/6 mouse showing a large endogenous T1-hyperintense peripheral lesion. (b) A corresponding 45  $\mu$ m formalin-fixed, sucrose cryoprotected

brain tissue section stained with hematoxylin and eosin showing a large peripheral melanotic lesion. There is no evidence of melanotic cell invasion of the outer layer of the cortex.

## **DISCUSSION:**

In general, internal carotid artery injection has been used to modulate the blood-brain-barrier (10-12), to test drug delivery to the central nervous system (13), as well as for inducing experimental models of brain metastasis (4,7,8). In the latter, mouse models of brain metastasis have been an essential tool for recapitulating many aspects of metastatic disease to the central nervous system. These experimental models have been crucial for preclinical studies aimed at better understanding the biology behind the dissemination of neoplastic cells to the brain. They also represent a great starting point for the preclinical evaluation of novel therapeutic regimens.

While surgically involved, this technique allows for the direct hematogenous introduction of cells to the brain with a low frequency of visceral spread – a complication typical of other injection routes such as the very popular tail vein injection, which typically induces more tumors in the lungs than in other organs (14-16). Therefore, injection into the internal carotid artery does provide a great advantage to studies focused on brain metastases.

We devised the use of gas anesthesia over injected for our internal carotid artery procedure. In the case where injectable anesthetic agents are considered, the weight of the mouse must be accurately determined using a calibrated per gram scale immediately prior to the procedure as weights can change over a 24 -hour period.

The cross section of the polyethylene tubing in step 23 can be further stretched manually at the tip to reduce its outer diameter (OD = 0.61 mm; 0.024”) to approximately of 0.25 mm (0.010”) using either a heat gun or heated oil. This procedure should be repeated several times until the desired tube thickness is achieved.

Great care should be exercised during blunt dissection of the neck. The proper identification of each important anatomical structure including trachea, major neck vessels and vagus nerve, is crucial for the success of the surgery. The placement of the two ligatures in the CCA and ECA (steps 18 and 19) ensured that the cells were only delivered to the ICA. The kink in the ICA as introduced in step 22 served to control bleeding from the CCA. Such bleeding can occur at this point in the surgery due to the regurgitation of blood from the ICA when the arteriotomy is performed on the vessel. In addition, thorough sterilization of the surgical field and instruments is critical to prevent post-operative infection and mouse demise.

Following surgery, the recovering mice were monitored closely for weight changes on a daily basis. Figure 2 illustrates a significant drop in mouse weight during the first three days following the surgery, prior to the gradual recovery of the animals. This drop in weight likely reflects poor feeding habits in the immediate postoperative period, and in turn, the high surgical strain that these mice are subjected to.

In our experience, the time required to inject one mouse was 35-60 minutes. The surgical mortality rate ranged from 20 to 25% among the 35 mice subjected to the procedure. One mouse was sacrificed post-operatively secondary to surgical site infection.

The detection of brain metastasis development in the animals was accomplished by magnetic resonance imaging (MRI). As shown in Figure 3, a T1-weighted sequence successfully detected peripheral metastasis as hyper intense lesions. This radiological feature on T1 is distinctive to melanotic tumors, and allows for three-dimensional image acquisition without the need of a contrast agent. Conversely, a MRI contrast agent will likely be required if a T1-sequence is chosen for non-melanotic tumors, provided that the tumor mass induces a disruption

of the blood-brain-barrier (17, 18). In the absence of either an endogenous T1 effect or contrast agent leakage, a less effective T2-weighted sequence will be required to visualize edema associated with the tumors (18).

Our results were validated using standard hematoxylin and eosin histological staining of brain tissue sections prepared at the end of our experiments. Notably, *in vivo* MR imaging provided us with a great advantage in allowing us to estimate tumor burden with time. With this technique we did not need to sacrifice the animals to evaluate the growing tumors as is required with traditional histological analysis. Each end user, however, must determine the best available readout of their experimental progress. Finally, we wish to stress that this technique can be applied to any mouse strain or cell-type. Furthermore, our procedure is amenable to the injection of experimental therapeutics, other chemicals and dyes if it is so required by the end user.

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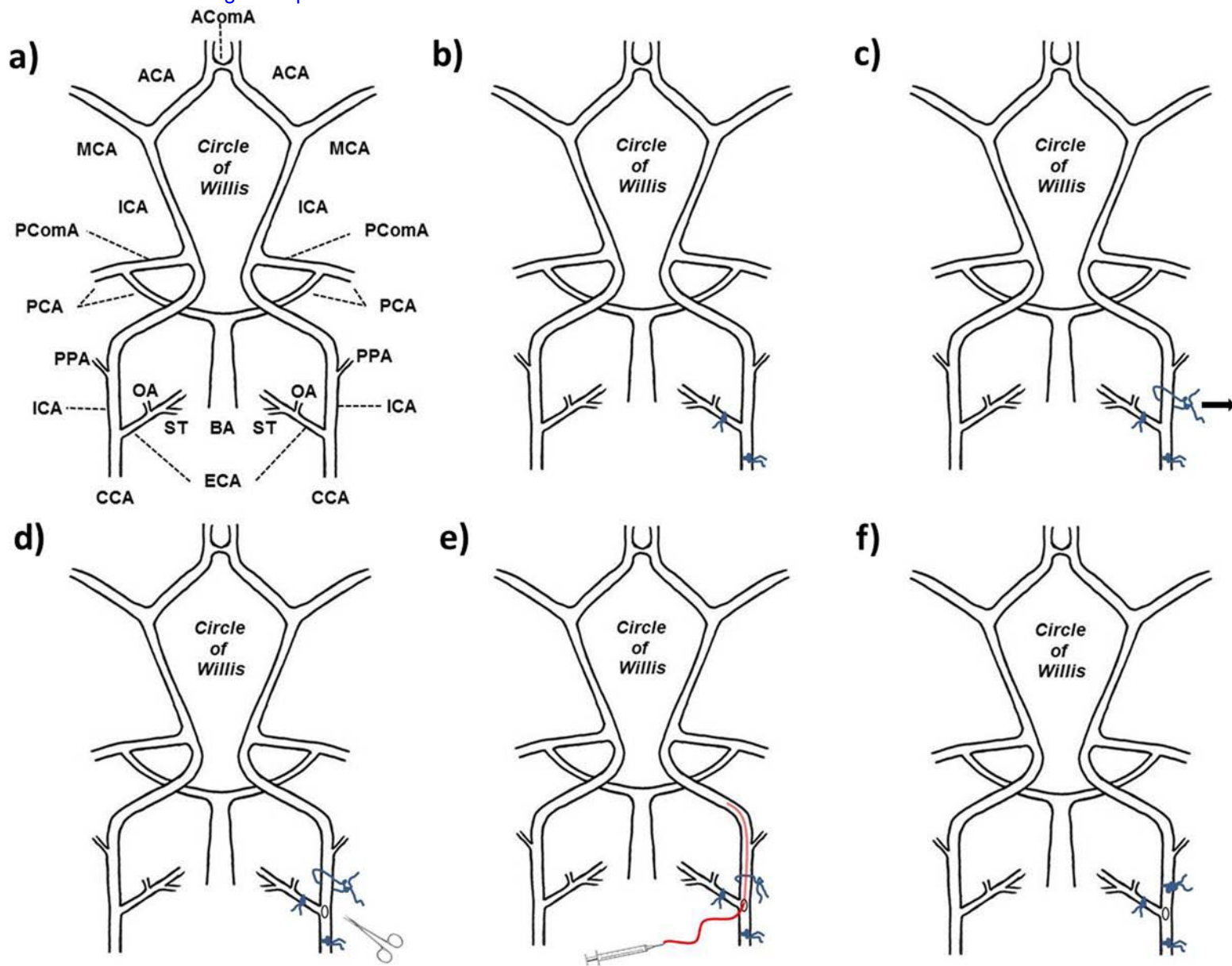
#### **DISCLOSURES:**

The authors have nothing to disclose.

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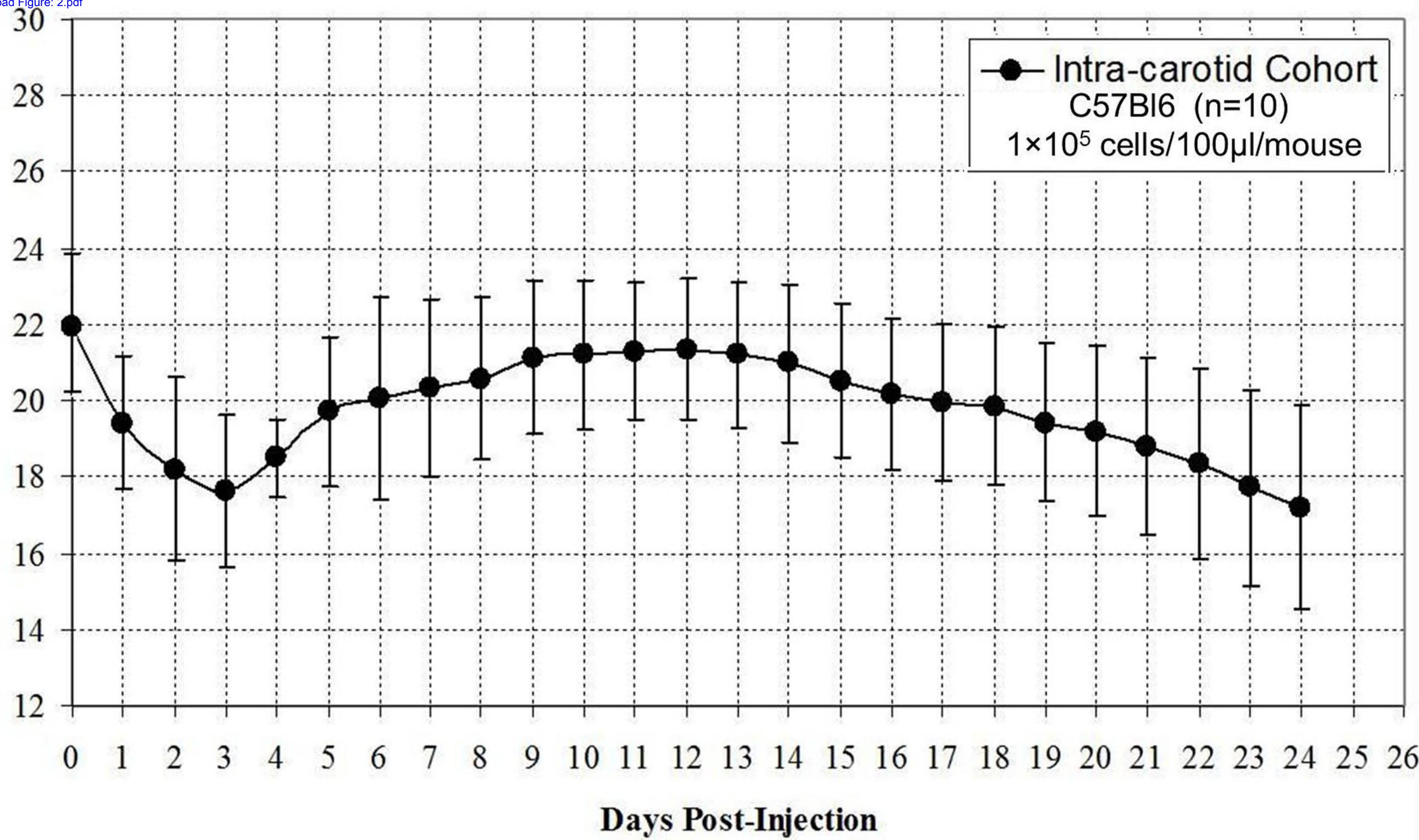
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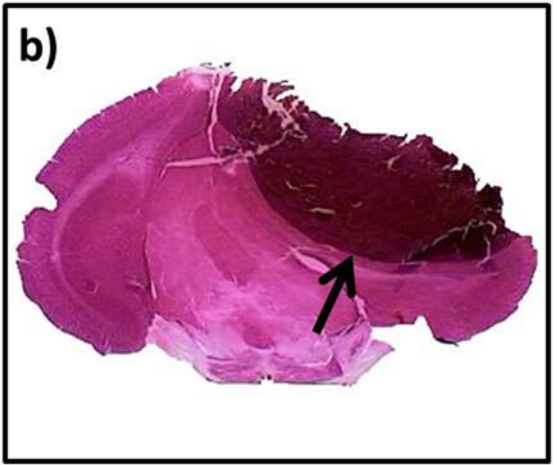
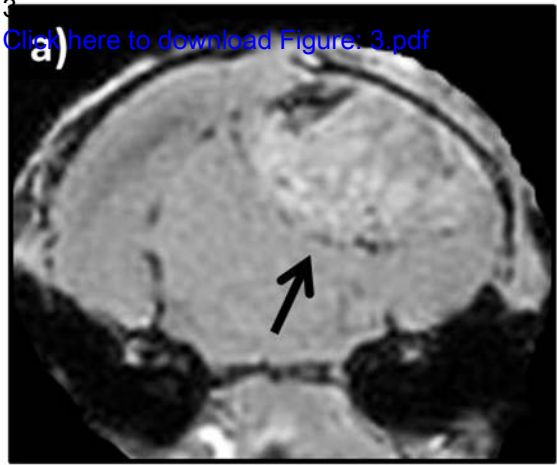
Mouse Body weight (g)

● Intra-carotid Cohort  
C57Bl6 (n=10)  
 $1 \times 10^5$  cells/100 $\mu$ l/mouse





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### AUTHOR:

Name:

Amr Morsi

Department:

Neurosurgery and Radiology

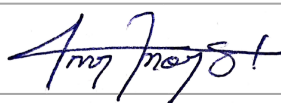
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Youssef Zaim Wadghiri, Ph.D.  
Assistant Professor of Radiology  
Director of Imaging Shared Resource

660 First Avenue, Room 444 New York, NY 10016

Phone: 212-263-3336

Facsimile: 212-263-7541

E-mail: wadghiri@med.nyu.edu

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Sincerely,

Youssef Zaim Wadghiri, Ph.D.

Assistant Professor  
Department of Radiology,  
New York University School of Medicine

