

Version 4: 041510

## ASSAY OF CA1 NEURONAL LOSS FOR GLOBAL BRAIN ISCHEMIA STUDIES

**Purpose:** This procedure is promulgated to assure an unbiased and uniform assay of tissue level outcome (specifically, CA1 neuronal loss) for global brain ischemia studies, as part of the CRL's commitment to the highest standards of scientific quality, integrity and clinical relevance.

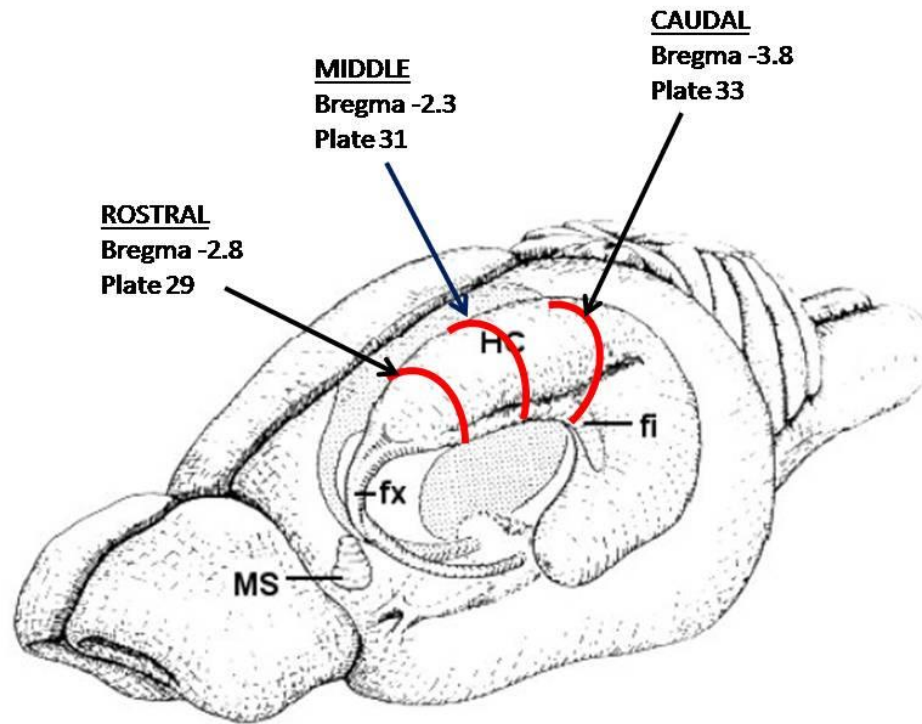
**Scope:** This protocol is to be followed for all studies incorporating tissue-level outcomes utilizing the global brain ischemia injury model.

**Review:** This protocol will be reviewed and updated annually or at the discretion of the Associate Director or Associate Chair for Research. Suggestions for improvement are to be forwarded to the Associate Director at [jsulliva@med.wayne.edu](mailto:jsulliva@med.wayne.edu).

**OVERVIEW:** This procedure comports generally with published approaches (in particular see Rod and Auer, *Stroke* 1992;23:725-732). The procedure uses whole brains subjected to transcardial perfusion fixation with ice cold saline and paraformaldehyde at sacrifice, followed by cryoprotection with sucrose, immersion fixation, and freezing in subzero isopentane (relevant SOPs pending; detailed procedures available on the secure portion of the lab website). The samples are sectioned on a cryostat in such a way as to obtain a representative sample of the hippocampus: rostral, middle and caudal sections are taken from stereotactically defined regions. Alternate sections are probed with  $\alpha$ -NeuN coupled to a DAB reporter or with cresyl violet, and prescribed areas of the hippocampal regions are photographed. The images are then assayed by counting of pyramidal neurons in the CA1 by multiple blinded observers. This SOP applies to the procedure beginning at the start of cryosectioning, and relies heavily on reference to Paxinos and Watson's *The Rat Brain in Stereotactic Coordinates*, available in the CRL Tissue Facility and hereafter referred to as "The Atlas."

1. **Cryosectioning.** Brains are mounted on the cryostat in the usual fashion, and are sectioned to the rostral edge of the hippocampus. The worker then takes 20  $\mu$ m sections through the hippocampus, sectioning exhaustively through regions indicated by Atlas plate 28 (Bregma -2.56) through plate 34 (Bregma - 4.16). When sections are taken, they are warm-adhered to subbed slides, and allowed to dry *outside the cryostat* for a minimum of 15 min. When the entire region from Atlas plates 28-34 has been sectioned, the slides are numbered and arranged sequentially in a slide box labeled only with animal's permanent designation (see randomization SOP)—indeed, this should be the only designation available to the technician at the time of sectioning. The slides will then be stored at -80 until used for staining.
2. **Neu-N Staining.** Neu-N staining will be carried out according to the standard laboratory protocol (SOP pending); counterstaining will be with DAB. *Alternate slides* will be used for DAB and cresyl violet, such that any slide in NeuN will have a closely corresponding slide in cresyl violet.
3. **Cresyl Violet Staining.** Cresyl violet staining will be carried out according to the standard laboratory protocol (SOP pending). Again, *Alternate slides* will be used for DAB and cresyl violet, such that any slide in cresyl violet will have a closely corresponding slide in NeuN.

4. **Slide selection.** The technician will select 3 cresyl violet *and* 3 Neu-N slides from each of the target zones (Rostral, Middle, Caudal) which demonstrate the least amount of confounding artifact (bubbles, chatter, tears, etc), and assure that these samples correspond to the appropriate region by comparison with the Atlas.

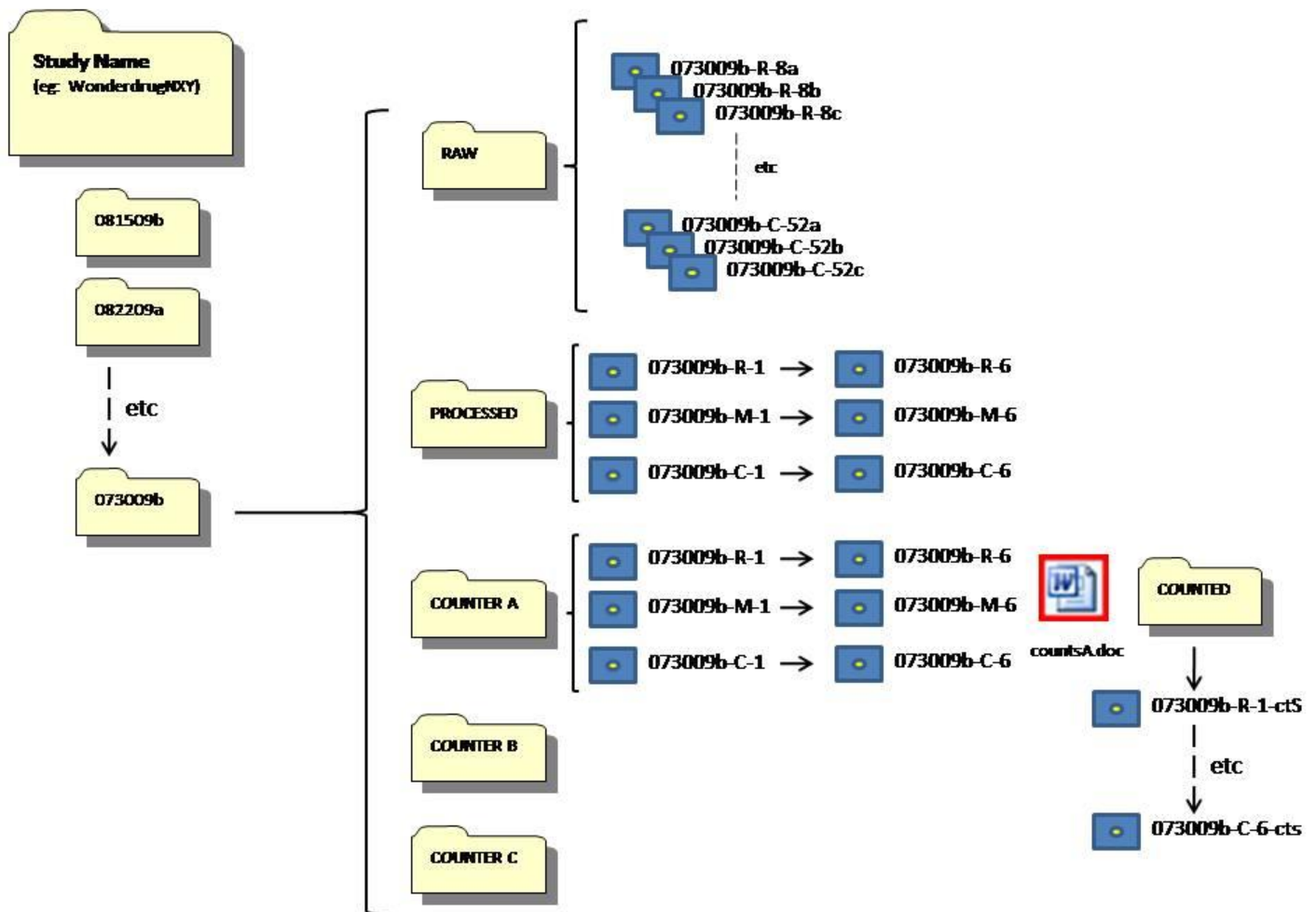


**Figure 1: Regions for hippocampal CA1 assay.** Indications general position of regions to be sampled for neuron counting, along with their stereotactic coordinates and corresponding plates in the Atlas. This figure is for illustrative purposes and orientation only; the Atlas is to be used to confirm that samples are taken from the correct region. Abbreviations: fx = fornix; fi = fimbria; HC = hippocampus; MS = medial septum. Modified from [Ikonen](#), 2001, after Amaral and Winter 1995.

5. **Photomicrography.**

- a. **CA1 area.** The technician will photograph the entire CA1 in overlapping images. The photomicrographs will be digitally stamped with a 100  $\mu$ m calibration bar.
- b. **Data management.** Photomicrographs will be saved in digital format on the Tissue Facility computer in a folder with the study name. The subfolder containing the images for a particular animal will be labeled with that animal's permanent designation (see randomization SOP). The individual images will be labeled with the animal's permanent designation (e.g., 10-11-09c), the region (R, M, C), the slide number, and the photograph number. For example, photomicrography of the entire CA1 in the rostral hippocampus may require 3 images from slide number 8. The 3 images would be labeled 10-11-09cR-8a, 10-11-09cR-8b, and 10-11-09cR-8c.

*Each time new data is added, the entire data set will be backed up to DVD or to a backup drive.*



**Figure 2: Data Management.** The figure demonstrates an example of how data is stored for CA1 counting studies. In this case, the folder for animal 073009b is exploded to show its contents. The subfolder “Raw” contains all the images taken at photomicrography, labeled with the animal designation, region, slide number, and photograph letter. For example, 073009b-R-52b is the second photo taken from slide 52, which is from the caudal region of animal 073009b. Composite images (montages) are stored in the folder “Processed” which should contain 9 composites (3 each of R, M and C regions) in NeuN and 9 in cresyl violet. Folders marked “Counter X” are simply copies of the “processed” folder with the addition of a text file for counters to record their results, and a new subfolder for storage of counted images. See SOP text for details.

6. **Image processing.** The images will be imported into imageJ and merged using the montage function. The resultant composite image will be saved in the same folder as the source images, with the permanent designation + region + comp + number. There should be 6 composite images for each region for each stain, so in our example the middle region would consist of 3 composite (montage) images labeled 10-11-09-M-1, 10-11-09-M-2, 10-11-09-M-3. The stain need not be designated, it is self-evident.
7. **Neuron counting.** Neuron counting will be carried out in a blinded fashion by at least two individuals, designated A, B, C...etc. One copy of the folder containing the processed images will be prepared for each counter, and will include a text file where the counter may record his/her counts for each region. Counting is performed in Image J using the “point selections” tool. A 300 µm region of the CA1 will be counted from left to right. The image is saved in the counter’s folder as a new image labeled with suffix “cts,” e.g., 10-11-09-M-3cts.

8. **Documentation.** The counter will modify the text document in his/her folder with the counts obtained and save the file.
9. **Data analysis.** The counts will be averaged separately by region, stain and counter. Accordingly, there will be an average counts for all the following:

|             | CRESYL       |              |              | NEU N        |              |              |
|-------------|--------------|--------------|--------------|--------------|--------------|--------------|
|             | COUNTER<br>A | COUNTER<br>B | COUNTER<br>C | COUNTER<br>A | COUNTER<br>B | COUNTER<br>C |
| Rostral CA1 |              |              |              |              |              |              |
| Middle CA1  |              |              |              |              |              |              |
| Caudal CA1  |              |              |              |              |              |              |

*The average counts for each region and each stain will be reported.* Using the data from the counters, a  $\kappa$ -value will be calculated and reported to document inter-observer variability. The data will be presented in accordance with the Data Analysis and Presentation SOP (forthcoming).

Approved:



Jonathon M. Sullivan MD, PhD  
 Associate Director  
 April 15, 2010