Fixing to collect? A validated brain tissue preservation method for mitigating the loss of neuroanatomical data in threatened biodiversity hotspots

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Introduction

Biodiversity hotspots, which harbor more endemic species than elsewhere on Earth, are increasingly threatened. There is a need to accelerate collection efforts in these regions before species become extinct. Vouchered specimens are fundamental to understand biodiversity because they represent near-permanent records of a species existence. The diverse data sets (e.g., ecology, ethology) generated from the collection of museum specimens affords investigators flexibility in how the data can later be used for a host of analytical approaches from molecular to macro-evolutionary scales.

A useful data source for understanding aspects of biodiversity is the brain. Characterizing the neuroanatomy of diverse species has been demonstrably useful in deciphering relationships between selection and adaptation that provide context about evolutionary mechanisms. Unfortunately, field methods used to preserve museum specimens are largely incompatible with neuroanatomical preservation. Moreover, most biodiversity hotspots are in areas with inadequate infrastructure for optimal preservation of brain tissue.

Here, we have developed a protocol that overcomes these challenges. We field-adapted two fixation methods commonly used in the lab: immersion and transcardial perfusion. We evaluated fixed tissues with iodine-enhanced X-ray micro-computed tomography (i-e µCT) and cytoarchitectonics. Our method is simple to implement in the field, requires few resources, and is extensible to researchers working in the world's most fragile ecosystems.

Methods

Trip details. A 58-day specimen collecting expedition to Central African Biodiversity Hotspots – Congo Basin and Albertine Rift.

Transcardial perfusion. Lizards were deeply sedated with isoflurane. The thoracic cavity was opened and thoracic wall removed to expose the heart. Lizards were exsanguinated from an incision to the right atrium, which also served to help flush the brain of blood. Syringes were used for successive injections of saline, followed by buffered formalin into the heart's apex at a constant hydrostatic pressure. In lab, fixative was ice-cold and conducted under a controlled hood.

Immersion fixation. Animals were sedated as described above, and manually decapitated between the second and third cervical vertebrae. Heads were immediately placed in buffered formalin. No immersion fixation occurred under lab conditions.

Storage. Brains were stored in vials containing a 12% sucrose-formalin solution. Infiltration of sucrose was confirmed when each brain lost buoyancy. Liquid levels in the vials were checked daily and replenished if low. Care was taken to avoid exposing the tissues to excessive heat, yet this was not entirely avoidable under field conditions in a tropical climate. In lab, dissected brains were immediately placed in cold storage overnight.

Freezing brains and histology. Brains were blotted dry and flash frozen with super-cooled hexane then stored at -80°C. Samples were embedded in Tissue-Tek OCT medium. Brains were cut into 30 µm-thick sections on the freezing stage of a Reichert-Jung OmE sliding microtome. Brain sections were collected in 24-well plates filled with anti-freeze cryoprotectant solution.

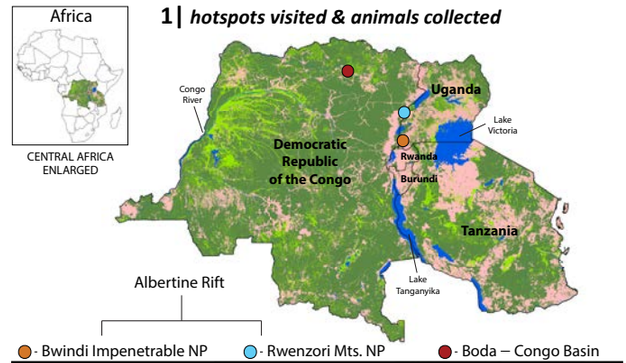
Nissl staining and imaging. Sections were rinsed in saline, mounted on gelatin-coated slides and dried overnight in a vacuum chamber. Series were stained with thionin. Stained tissues were examined with a Zeiss M2 AxioImager microscope and images were obtained using a cooled EXi Blue camera driven by Volocity Software.

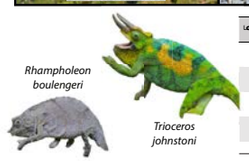
Tissue evaluations and statistical analyses. Blinded observers (3) scored tissue sections. To determine potential differences between methods, we ran generalized estimating equations (GEE) in R.

i-e X-ray µCT. Heads were transferred into aqueous Lugol's iodine (I_2KI) solution. Stained specimens were rinsed with water, blotted dry, sealed to prevent dehydration, and loaded into plastic mounting units for scanning. Specimens were µCT-scanned with a 2010 GE phoenix v|tome|x s240 high-resolution microfocus computed tomography system. Slices were assembled using VG Studio Max.

Results

1| *hotspots visited & animals collected*





Location	Species	SVL	TL	BW	Sex	Fixation, Duration	Duration w/o Cold Storage
1	*Trioceros johnstoni*	109	121	31.9	M	P, 58 min	54 d
2	*Trioceros johnstoni*	100	107	31.2	F	I, 18 min	51 d
2	*Rhampholeon boulengeri*	47	12	3.6	F	I, 25 min	51 d
2	*Rhampholeon boulengeri*	46	14	3.1	F	P, 33 min	51 d
3	*Agama sp.*	108	171	40.9	M	P, 46 min	21 d

Table 1. Species collected from hotspots and perfusion details.

2| *field method illustrated & supplies used*

Figure 1. (A) field lab; (B) field perfusion; (C-D) weight/size of animal; (E) pinned lizard on silicon mat w/ exposed thoracic cavity; (F) injection of fixative in heart; (G) exposed brain; (H) fixed brain in storage.

Table 2. Materials used for field procedures.

Item	Quantity	Supplier	Catalog #
1. Containers			
Falcon conical tube (50 ml)	5	Fisher	352070
field box	1	Plano Molding	131200
Fisherbrand polyethylene bottle (120 ml)	10	Fisher	02911952
2. Reagents and solutions			
10% formalin, sodium phosphate buffered	varied	See Methods	NA
Isosol (isoflurane, USP) (250 ml)	2	Vedco	
normal saline solution (250 ml)	2	Vedco	
sucrose (5 kg)	30 x 3 g	Sigma-Aldrich	S8501
3. Perfusion and dissecting instruments			
hypodermic needle (18 ga)	10	Nipro	AH+1825
syringe (3 cc)	3	Nipro	JD+03L
Dumont forceps (super fine tips)	1	FST	11252-00
Friedman-Pearson rongeurs (1 mm cup size)	1	FST	16020-14
interchangeable blades (angled)	10	FST	10035-15
Moria fine scissors (extra sharp)	1	FST	14370-22
pin (0.5 mm)	10	FST	26001-50
scalpel handle	1	FST	10003-12
spatula (metal)	1	FST	10090-13
surgical scissors (large)	1	FST	91402-14
Vannas spring scissors (2 mm cutting edge)	1	FST	15000-03
4. Miscellaneous supplies			
cotton ball (500/pack)	1	U.S. Cotton	
digital balance, battery-operated	1	Ohaus	
Parafilm "M" (2" x 250')	10 (strips)	Bemis	PM992
silicone mat	2	OXO	37210OV2
plastic ruler	1		

3| *method validation*

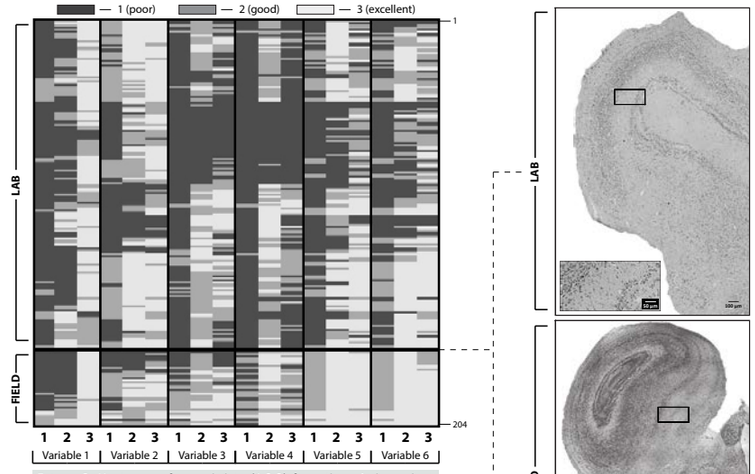


Figure 2. Heat map of scored data (1,2,3) from three independent observers (columns) across six variables (grouped columns) for tissue sections prepared with lab or field methods. Variables: 1 = blood; 2 = stain; 3 = edge integrity; 4 = center integrity; 5 = nuclei; 6 = lamination.

var	beta.Method	se.Method	z.Method	p.value.Method
Y1	0.3762	0.7023	0.5356	0.5922
Y2	-0.3755	0.7707	-0.4872	0.6261
Y3	1.5789	0.7633	2.0685	0.0386
Y4	7.0914	0.6314	11.2308	0.0000
Y5	26.9204	0.4919	54.7260	0.0000
Y6	23.5768	6.1940	3.8064	0.0001

Table 3. GEE analyses of scored data for each variable comparing methods (lab vs field).

Figure 3. Nissl stained tissues from lab and field perfusions.

4| *cytoarchitectonics*

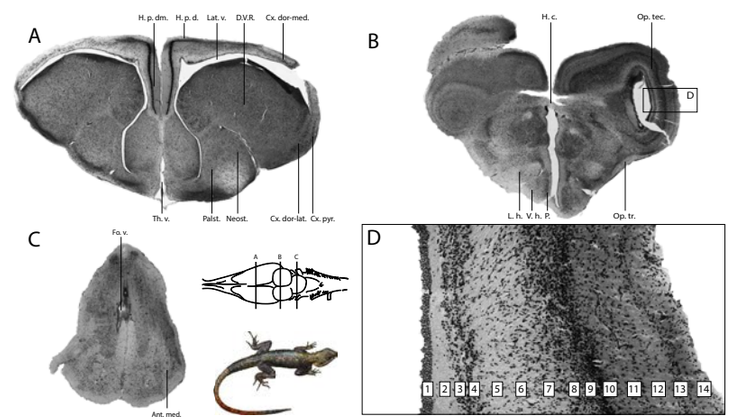


Figure 4. Rostral to caudal (A-C) Nissl stained coronal sections and labels correspnding to identifiable brain regions from a field-perfused brain of an Agamid lizard. (D) High-magnification photomicrograph of the optic tectum with 14 layers. Abbreviations: Ant. med. – Anterior medulla; Cx. dor-lat. – Cortex dorsolateralis; Cx. dor-med. – Cortex dorsomedialis; Cx. pyr. – Cortex pyriformis; D. V. R. – Dorsal Ventricular Ridge; Fo. v. – Fourth ventricle; H. c. – Habenular commissure; H. p. d. – Hippocampus, pars dorsalis; H. p. dm. – Hippocampus, pars dorsomedialis; L. h. – Lateral hypothalamus; Lat. v. – Lateral ventricle; Neost. – Neostriatum; Op. tr. – Optic tract; Op. tec. – Optic tectum; Palst. – Paleostriatum; P. – Periventricular hypothalamus; Th. v. – Third ventricle; V. h. Ventral hypothalamus.

5| *i-e µCT scans*

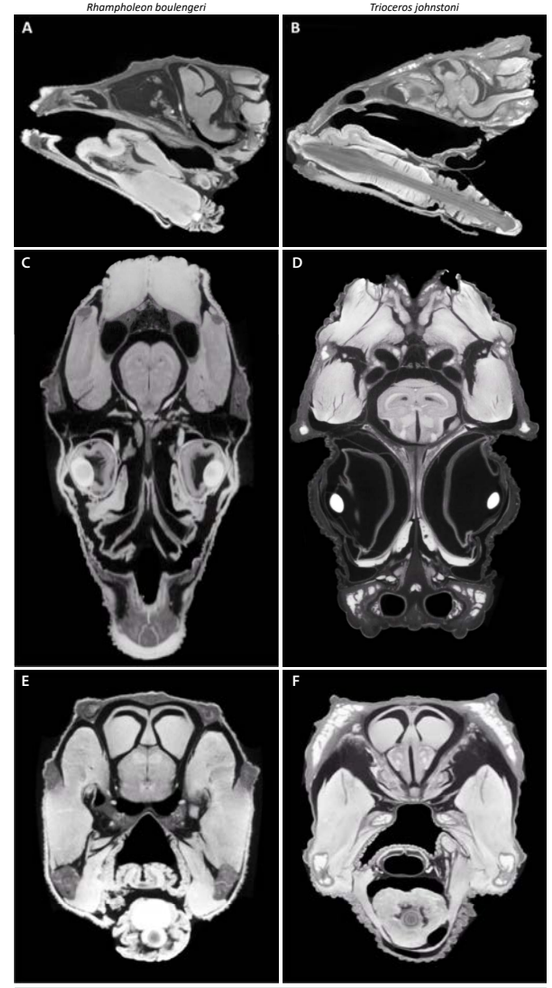


Figure 5. Sagittal, frontal, and transverse views of two chameleon species, *Rhampholeon boulengeri* (A,C,E) and *Trioceros johnstoni* (B,D,F).

Conclusions

We successfully validated a protocol for fixation of brain tissue in a completely mobile and long-term field setting, also environmental exposure did not negatively impact our tissues.

Our approach can (1) free researchers from laboratory limitations and (2) rescue perishable neuroanatomical information from threatened biodiversity hotspots.

Acknowledgments

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