Documenting brain diversity in field-caught lizards, from skull to cell: Initial development of a processing pipeline for top down, multi-scale structural analyses of a single brain by integrating specialized microcomputed tomography (diceCT), Nissl-based cytoarchitectonics, and immunohistochemistry

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Introduction

At the gross anatomical scale, computed tomography (CT) is a great tool for examining dense vertebrate tissues. However, traditional CT does not permit the visualization of non-mineralized soft tissues, such as the brain. The use of contrast agents for CT has helped to develop a means for differentiating a diversity of soft tissue types.

At the level of the neuron, studies that employ both cyto- and chemoarchitectonic approaches to staining are ideal to capture the full extent of phenotypic diversity displayed by cells within the brain. Multi-scale studies are necessary to elucidate the diversity of nervous tissues, and their spatial relationships to other tissue types.

In this study, we have developed an initial processing pipeline that involves field-fixed samples which we use to visualize neural circuitry from the cellular level to that of the entire brain in non-model species, and we discuss the potential for mapping brain interconnectedness in 3D across multiple scales of anatomy.

Methods

Trip details. During three independent expeditions (collectively spanning > 6 months) to Central Africa, a total of 10 lizard specimens representing 10 species from four genera were prepared for neuroanatomical investigations (Hughes *et al*., 2016)

Immersion fixation. Lizards were deeply sedated with isoflurane, and manually decapitated between the second and third cervical vertebrae. Heads were immediately placed in buffered formalin.

Storage. Heads were stored in 50 ml conical vials filled with a formalin-based fixative until processed for CT scanning.

CT 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.

DiceCT. Heads were transferred into aqueous Lugol's iodine (I_3KI) solution. Stained specimens were loaded into plastic mounting units and scanned as described above.

Brain dissection. The brain was dissected from the skull as described by Hughes *et al*. (2016), and immediately put into storage solution.

Freezing brains and histology. Brains were flash frozen, embedded in Tissue-Tek OCT medium, and stored at -80°C. Brains were cut into 30 µm-thick sections using a Reichert-Jung OmE sliding microtome. Brain sections were collected in wells with cryoprotectant solution.

Nissl staining and wide field imaging. Sections were mounted on gelatin-coated slides, dried overnight, and stained with thionin. Stained tissues were examined under bright field illumination using a Zeiss M2 AxioImager microscope. Wide field images were obtained using an EXi Blue camera driven by Volocity Software.

Immunohistochemistry and epifluorescence imaging. Sections were placed in a blocking solution, and then incubated in a cocktail of secondary antibodies. Sections were then reacted with fluorophore conjugates, counterstained, and mounted onto Superfrost slides. Immunostained tissues were visualized under epifluorescence illumination using the same the equipment described above.

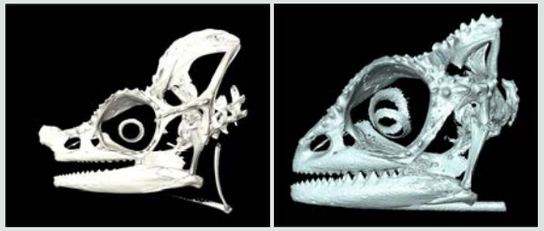
Results - Processing Pipeline

1. Sample Collection

- Collect fresh specimens
- Humanely euthanize animals
- Immersion fixation
- Post-fixation and long-term storage
- **Field protocol** (Hughes *et al*., 2016)

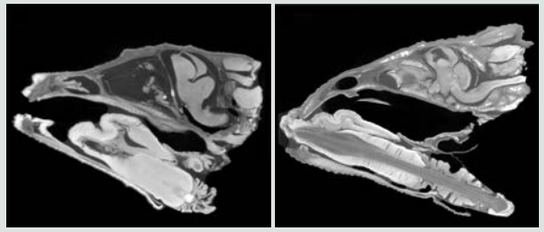
2. µCT Scanning

- Sample preparation
- Calibration
- Scanning
- Slice reconstruction
- Data output
- Segmentation
- **3D visualization**
- Measurements



3. diceCT Scanning

- Iodine staining
- Calibration
- Scanning
- **Slice reconstruction**
- Data output
- Segmentation
- 3D visualization
- Measurements

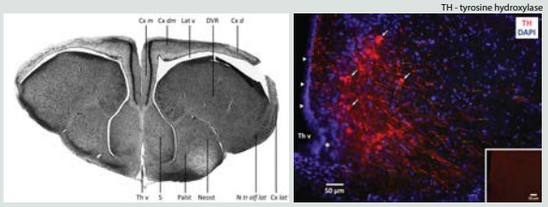


4. Neurohistology

- De-staining
- **Brain dissection**
- Sectioning
- Storage
- Nissl staining
- Immunostaining

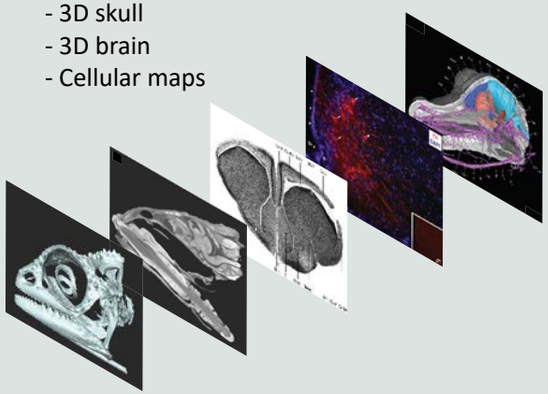
5. Brain Mapping

- **Photomicrography**
- Image manipulation
- Parcellation
- **Chemoarchitecture**
- **Cytoarchitecture**
- Mapping



6. Data Integration

- 3D skull
- 3D brain
- Cellular maps



Conclusions

- Through fully reversible contrast-enhancing agents for CT scanning (iodine for diceCT), researchers have the opportunity to examine the morphology of soft and bony tissues in the same specimen non destructively (Gignac & Kley, 2014).

- By rendering CT images in 3D, researchers can quantify morphology, including the volumetric dimensions of both the central and peripheral nervous systems, as well as their measurable relationships to bony features (Gignac *et al*., 2016).

- Our pipeline forms the foundation for future studies that aim to validate gross anatomical findings at the cellular level by implementing multiple imaging modalities, which sets up the potential for comprehensive mapping of brain interconnectedness across spatial scales.

- Other biological data, such as genetic sequences, can be integrated with the products of our pipeline to investigate the evolutionary implications derived from the structural variation in soft and hard tissue structures across species, and in turn, these findings could reveal phylogenetically informative characters that were not apparent from non-integrative, one-dimensional approaches.

- The data sets that can result from our pipeline are accessible to a remarkable breadth of data analysis platforms, and are easily combined with digital archives, which will be of great use to the biological community and undoubtedly help to expand the taxonomic coverage of online reference material.

References

Gignac PM, Kley NJ (2014) Iodine-enhanced micro-CT imaging: Methodological refinements for the study of soft-tissue anatomy of post-embryonic vertebrates. *J Exp Zool B Mol Dev Evol* 322: 166–176.

Gignac PM, *et al* (2016) Diffusible iodine-based contrast-enhanced computed tomography (diceCT): An emerging tool for rapid, high-resolution, 3-D imaging of metazoan soft tissues. *J Anat* 228: 889–909.

Hughes DF, *et al* (2016) Rescuing perishable neuroanatomical information from a threatened biodiversity hotspot: Remote field methods for brain tissue preservation validated by cytoarchitectonic analysis, immunohistochemistry, and X-ray microcomputed tomography. *PLoS ONE* 11: e0155824.