Neuropathological work up and whole slide image analysis of human brain tissue

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ABSTRACT

The National Institute of Aging – Alzheimer's Association (NIA-AA) have established criteria for the neuropathological assessment and diagnosis of Alzheimer's disease which dictate specific regions of the brain to be examined for that purpose (1). The histological characterization of the pathologic peptides in the relevant regions of brain is critical to properly diagnosis neurodegenerative disease and thus placing the utility of the research tissue in the proper context. As new consensus criteria emerge, the histologic workup evolves to keep pace. This has grown to include a more thorough evaluation for the histopathologic hallmarks of chronic repetitive neurotrauma, staging of limbic-predominant age-related TDP-43 (LATE) pathology, and assessments of age-related tau pathology in neurons and glia (i.e.. PART and ARTAG). A standardized workup focused on general neuropathology and neurodegenerative disease according to NIA-AA guidelines is applied to all cases, with additional modular staining programs developed to target additional pathologies or networks. A complete list of the routine brain regions sampled, and standard staining techniques, used is provided in Table 1.

The following standard protocol used in our laboratory covers the NIA-AA criteria for pathological diagnostics and achieves the high quality necessary to perform consistent digital image analyses. The value of histologic stains can be increased by quantitative analysis of the pathologic peptide burden, which has great potential to identify, extract, and quantify tissue features in greater detail than the standard pathological assessment (2). Slide scanning analyses develop precise assessments of structural changes in histologic sections across a wide range of brain regions, which can inform understanding of the basis of selective vulnerability in neurodegenerative disease. Such analysis can identify frequency and percent area involved by pathologic peptides, neuroinflammation, and neurodegeneration. Quantitative neuropathology is necessary to understand how pathologic burden differs across brain regions in association with cognitive function or biomarker studies, as similarly classified individuals can vary significantly.

The digital pathology concept refers to the use of computer work-stations to view the whole slide image (WSI) for sharing, teaching and primary reporting diagnostics (3).

MATERIALS

Standard neuropathological workup

1. Glass slides.
2. Xylene.
3. Ethanol de- or re-hydration series, 100%, 95%, 70%, and 50% in ddH_2O.
4. *Tris-buffered saline*(TBST); for 20 L: 700.8 g NaCl, 484.4 g Tris-HCL, in ddH_2O; adjust pH to 7.2 with NaOH or HCl, then add 20 mL Tween-20.
5. Optional: NexGenDecloaking Chamber™and 1X Diva Decloaker solution, or equivalentheat-induced epitope retrieval (HIER) system.
6. Hematoxylin/eosin with Luxol Fast Blue stain (H&E/LFB): Luxol fast blue 0.1g. 95%Ethanol 100ml, 10% acetic acid 0.5ml, 200 ml of each hematoxylin, bluing solution, eosin y w/ phloxine)
7. Bielschowsky silver stain.
8. Coverslips.
9. Mounting medium.
10. Slide boxes.
11. Leica Microtome.
12. Water bath.
13. High-profile microtome blades.
14. Biocare intelliPATH (semi-automated staining device), or equivalent.

Digital neuropathology

1. Slide scanner (Aperio AT2 Leica, or equivalent).
2. Cloud-based file storage system (*e.g.,*Amazon Web Services, or equivalent).
3. *HALO* image analysis platform (Indica Labs).

Neuropathological work up

1 Cut 5-µm-thick sections fromparaffin-embedded tissue blocks using a microtome.

2 Form a ribbon of tissue using the microtome and a blade to cut the tissue.

3 Transfer ribbon to a 2-7°C water bath

4 Pick up the tissue from the water bath using a glass slide.

5 Bake at 16°C for 10 min to 24 h, to adhere tissue to the slide and melt the paraffin

6 To remove paraffin, immerse slides sequentially in a series of 3 vessels containing xylene, for 3 min each. Change to fresh xylene every 120-150 slides.

7 Rehydrate in a graded ethanol series (3X at 100%, 2X at 95% and 1X with ddH_20) for 3 min per wash. Change to fresh ethanol every 120-150 slides.

8 Perform immunohistochemical (IHC) staining on a subset of slides (the list of antibodies for both single and duplex stains and their specific protocols are included in table 1):

8.1 Use heat-induced epitope retrieval (HIER) at 110°C for 15 min to increase antigen-antibody binding and thereby enhance the IHC signal

8.2 Cool the slides for 10 min at room temperature

8.3 Wash the slides twice with TBST for 5 min each

8.4 Place the slides in a Biocare intelipath (or equivalent) semi-automated staining device, following the manufacturer's protocols, and using reagents recommended for the antibody of choice.

8.5 Once IHC staining is complete, remove the slides and wash for 3 min in TBST, then dehydrate in an ethanol series (70% once, 96% once, then 3 times in fresh 100% ethanol) for 3 min each, then immerse in xylene sequentially in a series of 3 containers, for 3 min each. Change the ethanol and xylene solutions every 120-150 slides.

8.6 Apply mounting medium and a coverslip to each slide

8.7 Let dry, then store in slide boxes at room temperature

9 Perform histochemical staining (manually) using standard protocols for:

9.1 Hematoxylin and eosin with Luxol fast blue stain (H&E/LFB); and

9.2 Modified Bielschowsky silver stain

9.3 Once IHC staining is complete, dehydrate in an ethanol series (70% once, 96% once, then 3 times in fresh 100% ethanol) for 3 min each, then immerse in xylene sequentially in a series of 3 containers, for 3 min each. Change the ethanol and xylene solutions every 120-150 slides.

9.4 Upon completion, apply mounting medium and coverslips to slides. Store in slide boxes for examination by a neuropathologist (see below).

10 Perform microscopic examination of the stained slides

11 Record all neuropathologic data points in a database

Table 1 - Antibodies and antigen retrieval protocols

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	A	B	C	D	E	F
	PRIMARY ANTIBODY	**CLONE/ HOST**	**COMPANY**	**CATALOG NUMBER**	**DILUTION**	**PRETREATMENT**
	Alpha Synuclein	LB509/Mouse	Invitrogen	180215	1:200	Proteinase -K (10 min)
	Beta Amyloid	6 E10/Mouse	Biolegend	803003	1:1200	Diva/Decloaker (110°C-15min)
	Anti-Huntington's Protein	mEM48/ Mouse	Millipore	MAB5374	1:50	Diva/Decloaker (110°C-15min)
	Notch 3/N3ECD	1 E4/Mouse	Millipore	MABC594	1:2000	Diva/Decloaker (110°C-15min)
	SQSTM1/p62	2C11/Mouse	Abcam	ab56416	1:500	Diva/Decloaker (110°C-15min)
	PHF-Tau	AT8/Mouse	Thermo Fisher	MN1020	1:1000	Diva/Decloaker (110°C-15min) or ER 1 10 min
	Anti-Prion Protein	3F4/Mouse	Millipore	MAB1562	1:50	ER1 (20 min)
	TAU RD3	8E6/C11/ Mouse	Millipore	05-803	1:2500	Diva/Decloaker (110°C-15min)
	TAU RD4	1E1/A6/ Mouse	Millipore	05-804	1:400	Diva/Decloaker (110°C-15min)
	phosTDP-43 (Ser409/Ser410)	ID3/Rat	Biolegend	829901	1:1000	Diva/Decloaker (110°C-15min)
	TDP-43 (TARDBP)	Rabbit	Protein Tech Group	10782-2-AP	1:500	Diva/Decloaker (110°C-15min)
	Ubiquitin	Ubi-1/Mouse	Millipore	MAB1510	1:30,000	Diva/Decloaker (110°C-15min)

Digital Neuropathology

13 Obtain a whole slide image

13.1 Clean slides with 70% ethanol and Kimwipes to remove any particles which might cast shadows on the underlying tissue sections

13.2 Scan the majority of slides at 20x magnification, which is considered suitable for manual histochemical and IHC viewing and image analysis (Kumar, 2020), and is more cost effective compared to 40x due to the reduced storage space and scanning time required.

13.3 Perform quality controls measures to assess for positivity in control slides, IHC artifacts (non-tissue or cell specific-DAB substrate stains, bubbles, unstained areas) and features which might impede digital analysis (dark shadows, folds, or tears).

14 Import images to image storage in cloud-based servers

14.1 These can be set up with individual user accounts to control access to groups of images and can be expanded as needed

14.2 Ensure HIPAA compliancy and compatibility with image analysis software

15 Begin visualization and analysis by annotating brain regions and objects of interest.

16 Use appropriate image analysis software (such as HALO® v.3.4.2986 (Indica labs, Albuquerque, New Mexico, USA)) modules to identify, extract and quantify neuropathologic features

Table 2 - HALO modules used and type of data obtained fro...

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Module	Type of Data obtained by ROI	Unit
Area quantification (up to 3 markers)	Percentage of Area by marker	Percentage
Object Colocalization (up to 2 markers)	Total ROI Area	mm2
	Total object 1 and 2 count	µm2
	Total object 1 and 2 area	qty
	Object 1 and 2 percent area	Percentage
	Total object colocalized area	qty
	Percent of object 1 and 2 colocalized	Percentage
	Area of positive stain by object	µm2
	Colocalized area by object	µm2
	Percent of colocalized area of each marker by object	Percentage
Multiplex IHC (up to 5 markers)	Total cells by marker	qty
	Area of positive stain in nucleus by marker and by object	µm2
	Area of positive stain in cytoplasm by marker and by object	µm2
	Area of positive stain in membrane by marker and by object	µm2