

Visible Human 2.0 – the Next Generation

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The National Library of Medicine has initiated the development of new anatomical methods and techniques for the acquisition of higher resolution data sets, aiming to address the anatomical artifacts encountered in the development of the Visible Human Male and Female and to insure enhanced detection of structures, providing data in greater depth and breadth. Given this framework, we acquired a complete data set of the head and neck. CT and MR scans were also obtained with registration hardware inserted prior to imaging. The arterial and venous systems were injected with colorized araldite-F. After freezing, axial cryosectioning and digital photography at 147 microns/voxel resolution was performed. Two slabs of the specimen were acquired with a special tissue harvesting technique. The resulting tissue slices of the whole specimen were stained for different tissue types. The resulting histological material was then scanned at a 60x magnification using the Virtual Slice technology at 2 microns/pixel resolution (each slide approximately 75,000 x 100,000 pixels).

In this data set, for the first time anatomy is presented as a continuum from a radiologic granularity of 1 mm/voxel, to a macroscopic resolution of .147 mm/voxel, to microscopic resolution of 2 microns/pixel. The hiatus between gross anatomy and histology has been assumed insurmountable, and until the present time this gap was bridged by extrapolating findings on minute samples. The availability of anatomical data with the fidelity presented will render it possible to perform a seamless study of whole organs at a cellular level and provide a testbed for the validation of histological estimation techniques. A future complete Visible Human created from data acquired at a cellular resolution, aside from its daunting size, will open new possibilities in multiple directions in medical research and simulation.

The landmark Visible Human Male (VHM) and Visible Human Female (VHF) data sets [1] were released by the National Library of Medicine (NLM) in 1994 and 1995 respectively. Throughout the ensuing years, the data was extensively explored, used in applications by educators, researchers in numerous specialties medicine, basic biomedical research as well as various branches of computer science. The data sets proved an inexhaustible source of medical information. Drawing on the experience gained from the broad range of users and applications, the National Library of Medicine has contracted two independent research groups to investigate and develop new methods and techniques, that will address the anatomical artifacts and problems apparent in the VHM and VHF data and will provide anatomical information of greater depth and breadth. In this paper we present the results of our group. Initially planned as a project of technical feasibility, the work resulted in a complete data set of the head and neck anatomy. It is available from the NLM under the same conditions as the VHM and VHF data sets.

In addition to having addressed the concerns that prompted this initiative, we also present an important addition to the initial radiology-cryosection pair. The histological sections of the specimen will open new possibilities in many applications.



Figure 1. Detail of a cryosection. Note the filling of the small blood vessels, grey matter/white matter contrast and the distinct layers of the tentorium cerebri encasing blood vessels.

Materials and Methods

1. Specimen selection. The specimen was selected through the body donation program of the University Medical Centre in Utrecht, based on age, size and pathology. The choice was made for a Caucasian male 66 years of age, without known pathology in the head and neck region.

2. Fixation. The fixation of the body was done by injecting of formalin 4% into the femoral artery. The femoral vein was opened to evacuate the blood. The perfusion was considered complete when clear fluid came out of the femoral vein.

The specimen was frozen at -20°C and a band saw (Model 4210, Reich Specialmaschinen GmbH, Germany) with a Uddeholm blade (BS Super 3/2915x20x6x0.6 mm) was used to trim the frozen specimen to a block of 230x250x150 mm, taking into account the direction of sectioning, the limitations of the cryotome, allowing room for embedding medium around the specimen. The frozen specimen was then thawed and rinsed well in running tap water to remove fixation fluids or any other extractable materials. **3. Vascular filling.** The right common carotid artery was cannulated and connected to a reservoir containing the following mixture: 20 units of volume Araldite F with 7.5% Microlith-T, 60 units of volume of diluener DY 026 SP, and 45 units of volume of hardener HY 2967. Although the dynamic viscosity was very low, the surface tension of the liquid would still have prevented it from filling the smaller vessels. In order to lower the surface tension 0.05 ml of liquid soap was added to 160 ml of the mixture. The reservoir containing this mixture was pressurized with an air pump at around 150 mm HG, injecting the vascular tree. When the mixture emerged from the left common carotid artery and the vertebral arteries these vessels

were clamped. The vascular filling was considered to be completed when the level in the reservoir remained stationary. The procedure was repeated for the venous system [2]

3. Demineralization of the teeth. The hardest material in the specimen was the enamel of the teeth. The teeth tend to chip during cryosectioning, and the section of these structures are usually unsatisfactory; in addition, the enamel dulls the microtome blade. The following technique was used to demineralize the enamel: cottonwool soaked with formic acid was packed around the teeth. Over 48 hours, every 2 hours fresh fluid was applied to the cottonwool to keep the concentration at the required level.

4. Fiducial Markers. In order to insure a good registration between the imaging modalities, we inserted three aluminum alloy markers, which did not scatter X-rays, yet were visible on magnetic resonance imaging (MRI). Two such markers were inserted into the temporal bones on each side, approximately at the same level, and the third into the mandible.

5. Radiologic imaging. Computed axial tomograph (CAT) images were obtained at 0.5 mm interval (contiguous slices). MRI at 3 mm contiguous slice thickness was acquired

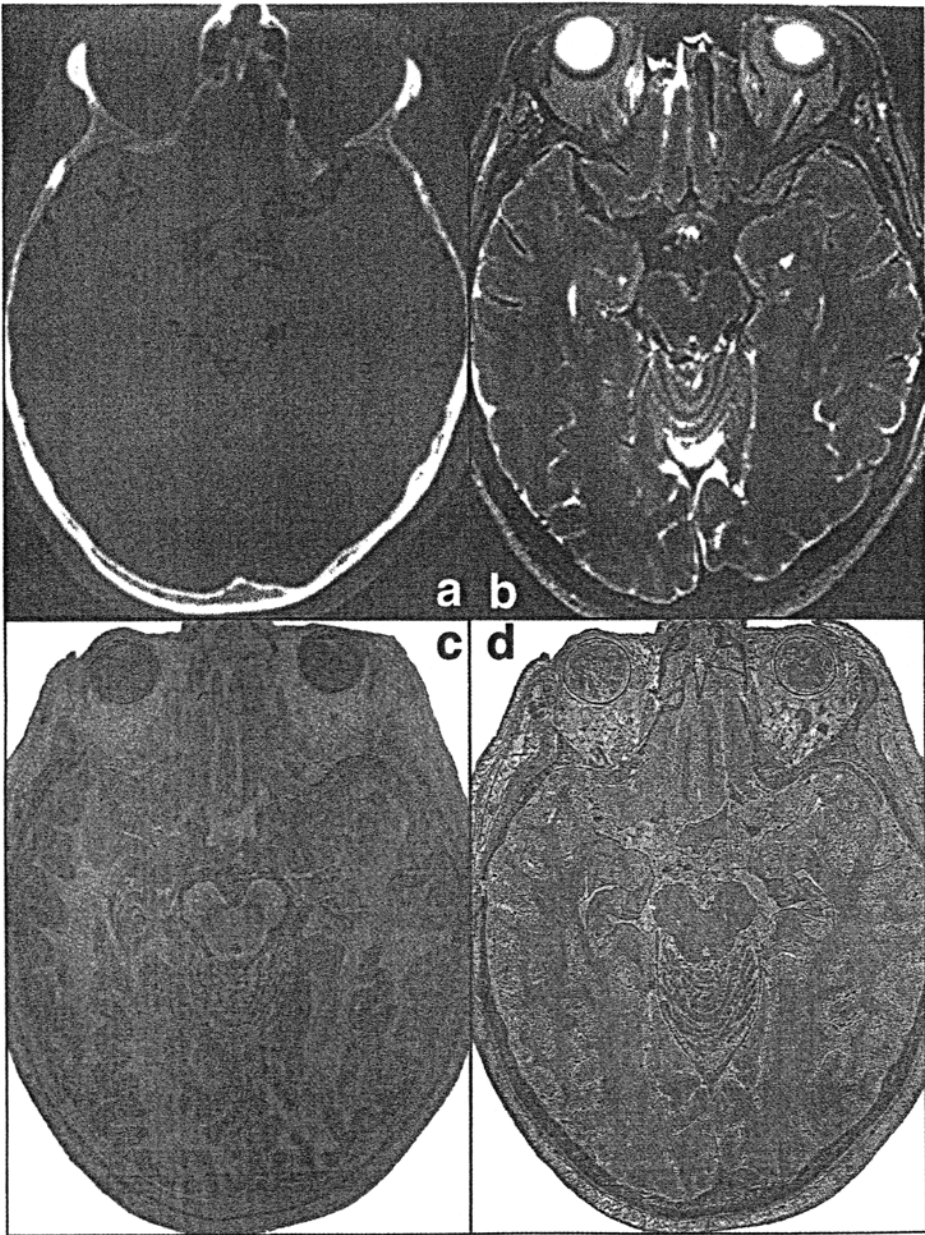


Figure 2. Visible Human 2.0. a -CAT scan. **b**-MRI scan. **c**-cryosection. **d**-histological stained section. Note the good alignment between modalities, before post-processing.

with T1, T2 and Pd sequences. Next, the specimen was embedded and frozen.

6. Embedding. We used high viscosity carboxymethyl-cellulose (CMC) gel (BHD Chemicals Ltd.). In order to obtain good infiltration we used two impregnation baths, the first with a solution of 0.5% CMC gel and the second with 1.0% CMC gel, over four days.

8. Freezing. The mold was cooled by placing the base of the microtome stage in a shallow pool of liquid nitrogen (-196°C) The specimen was positioned in the mold with the temporal fiducial markers level and freezing commenced, with liquid nitrogen kept at

constant level for 2 hours. The block was then placed in the cryomicrotome (L.K.B. PMV 450MP) at -25°C , for further freezing over the next 24 hours.

7. Cryosectioning. Sections 21 μm thick were cryoplaned with a high quality steel knife (L.K.B. type M-140-04) at a speed of about 3.0 m/min. After 7 sectioning cycles amounting to 147 μm a digital photograph of the surface of the block was taken, at a resolution of 1525X1146 pixels or 147 $\mu\text{m}/\text{pixel}$. The entire specimen was captured on 1481 digital images. In addition to the digital images, a few random samples and the entire slab of tissue harvested on tape were photographed with a medium format camera on conventional color reversal film and digitized at 65 $\mu\text{m}/\text{pixel}$ resolution. See Figure 1 for a detail of a section captured with conventional photography.

8. On tape histological sections. On tape sections were collected in the following manner: adhesive tape was laid onto the cryoplaned surface and gently pressed down with a plastic block (130x35x20 mm) in front of the knife. A contiguous block of 69 samples was harvested at 20 μm thickness and another block of 40 samples was harvested at 30 μm with a slightly different technique, using the Macro Tape Transfer System (MTTS Instrumedics, Inc., Hackensack, NJ). The on tape sections were air dried at room temperature before staining.

9. Staining . In order to obtain adequate staining and to protect the tape, we used a fast one-step dye bath and a non-aggressive rinsing procedure. The original Cason modification of Mallory's trichrome combined all the Mallory stains in the same solution. Applied to on tape sections, this solution produced heavy staining of the tape and dyes ran easily in the rinsing baths. Our dye bath uses slightly modified concentrations: 0.5 g of phosphotungstic acid (E. Merck, Darmstadt), 0.1 g of orange G (C.I. 16230), 0.1 g of aniline blue (C.I. 42755), 0.1 g of acid fuchsin (C.I. 42685) (all products were obtained from Gurr, BDH Chemicals, Ltd.) in 100 ml of distilled water. The solution was filtered prior to staining [3]. An additional number of sections were stained with cresylviolet and luxol fast blue. The on tape cryosections were stained for 2 minutes and the rinsed for 45 seconds in running tap water followed by a brief rinse in 70% ethanol (10 sec) and 96% ethanol (5 sec). For support, the non-adhesive side of the wet tape was then wrapped part way around the polyvinyl chloride cylinder with a circumference greater than the length of the tape. This kept the section flat and well stretched while excess alcohol was evaporated with an air blower. The on tape section was then rolled onto a piece of thin white cardboard. The section was thus supported on one side by the cardboard and protected on the other side by the tape. Sections and cardboard were immediately placed under pressure for 24 hr to prevent the edges from curling. The section-cardboard combination can be studied using reflected light up to magnifications of 20 times. The useful magnification is limited by the reflectivity of the tape covering the section.

Two digital versions of the on tape cryosections were made. The entire batch of sections affixed on cardboard was scanned on a flatbed scanner at 900 dpi resolution. Samples of the sections affixed on cardboard and the sections affixed on glass, harvested with the MTTs were scanned with the Virtual Slice method, described below.

10. Virtual Slides. Each virtual slide of the tissue is an image montage composed of the numerous contiguous high-magnification fields-of-view from a microscope. Viewing these virtual slides permits lossless zooming, up to the magnification at which the virtual slide is acquired. The hardware configuration used for virtual slide acquisition was a computer microscopy system comprised of a research microscope, high-resolution motorized stage, digital CCD camera, and PC. The synchronization of the stage, camera, and computer is orchestrated by the NeuroLucida™ software with Virtual Slice module (MicroBrightField, Inc, Williston, VT). NeuroLucida automatically scans the tissue by moving the motorized stage in a boustrophedon pattern, acquiring images for each field-of-view within a defined region.

At the end of the scan, the images are automatically stitched into a single high-

resolution montage. This montage undergoes a subsequent digital compression using a lossless JPEG algorithm, which considerably reduces the final output size. The output file was written in the Zoomify file format. [4].

Each scan was performed using a Zeiss Axioskop II microscope fitted with a Ludl stage and a DVC 1300x1024 digital camera. To illuminate sections that were mounted on paper, we used a combination of transmitted light and incident light from a fiber optic light source. Illumination of sections mounted on glass slides was performed using only transmitted light.

Attempting to image large regions of tissue posed a multitude of technical difficulties which had to be overcome before the production of satisfactory virtual slides could be achieved. One such problem was keeping the tissue in focus across the entire scan while the tissue was perfectly in focus within a single field-of-view, there was enough variation in the thickness of the slide that tissue near the edges of the larger scans tended to be out of focus. This was less evident at the lower magnifications because depth-of-focus is inversely proportional to objective magnification.

Uneven illumination posed an even greater challenge to be overcome. To counter this problem a background correction algorithm was utilized to correct individual images in the montage for variances due to uneven illumination. Even when great care was taken to illuminate the tissue evenly, the final montages had a subtle checkered appearance due to chromatic variance, which is especially evident on the lowest power scans. We are currently investigating several solutions to the chromatic variation.

Among the magnifications used to create the virtual slides we tried objective lenses ranging from 1.25x to 40x. The best results we obtained were with a 5x lens. At higher magnifications the narrower depth-of-field reduced the overall quality of the images. A 20x objective lens was used to image the section of the whole cerebellum with good success. Due to the size of the tissue, two scans with a 5x objective were performed separately on the front and the rear parts of the head. Table 1. gives an overview of the file sizes at different magnifications and in different file formats.

Table 1. File sizes of virtual slides

Region	Objective lens	Absolute Magnification	Fields-of view	Uncompressed file size	Compressed file size
Cerebellum	20x	~500x	24,000	96GB	2.4GB
Front of head	5x	~125x	8,250	33GB	1.1GB
Back of head	5x	~125x	9,300	37GB	1,2GB

Discussion

The technique that we have used and described above has corrected all the main shortcomings of the VHM and VHF: in our data set there is no noticeable post-mortem swelling of the central nervous system (CNS) and the subarachnoid space is clearly visible; the white matter/grey matter contrast is greatly improved; the vessels are filled to a sub-millimeter level, with clear differentiation of the arteries and veins; the teeth were adequately cryoplaned. In addition, we provided external fiducials that will insure improved registration of the cryosections with the radiologic data. This will greatly benefit data analysis and segmentation will become transferable between imaging modalities. We also provided data improved image capture by conventional conventional photography.

The principal novelty of our approach consists in the addition of stained histological slides in digital format, obtained with the *Virtual Slide* technology. We provided a sizeable sample of histological slides, adequate for the assessment of the benefits of this technique:

References

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