ALLEN INSTITUTE for BRAIN SCIENCE

TECHNICAL WHITE PAPER: HUMAN CORTEX STUDY

Project Overview

The Allen Institute for Brain Science Human Cortex Study presents the general landscape of gene expression patterns across different cortical regions and human individuals. Gene expression is detected using colorimetric *in situ* hybridization to provide cellular level resolution in non-diseased adult human neocortex. The data are publicly accessible via a Web-based application that allows viewing of indexed imaged sets searchable by gene, cortical area, specimen and donor characteristics.

The initial launch of the Web site (<u>http://humancortex.alleninstitute.org</u>) in mid-November 2007 included image data for approximately 500 genes in visual cortex and temporal cortex of at least one subject. Data releases throughout the course of the project will expand the dataset to include additional genes, cortical regions and individuals.

Genes represented in this dataset comprise several categories of broad scientific and clinical interest: cortical cell type markers, gene families important to neural function, disease-related genes and genes important in the comparative genomics field.

Pipeline Overview

High-throughput processes for generation of ISH-based gene expression data were developed at the Allen Institute for Brain Science for the production of the Allen Brain Atlas – Mouse Brain (<u>http://www.brain-map.org</u>), a genome-wide atlas of gene expression in the mouse brain (1). The process, equipment and workflow for generation of gene expression data in human cortex closely follows that described for generation of the Allen Brain Atlas – Mouse Brain; see reference (1), Supplemental Methods 1) with some adaptation to manage specific challenges posed by working with human tissue. For example, processes to characterize tissue and RNA quality were added, and image acquisition systems and data processing capacity were modified to accommodate larger tissue sections.

In addition to changes in laboratory production processes, the Laboratory Information Management System (LIMS) was updated to accommodate information management needs specific to human tissue samples. The LIMS is also used to view raw image data to check image quality after initial image acquisition.

Overall workflow for this project is shown in Figure 1. Briefly, validated tissue and probes are coordinated in work packets, where each packet comprises tissue and probes that progress as a discrete unit through sectioning, ISH, image acquisition and processing. Once data pass image and data quality control, images are ready for public display. Internal data analysis guides prioritization of genes for subsequent work packets. This workflow enables the systematic generation of data for all genes in multiple regions in multiple cases with equivalent male and female representation.

All processes associated with data production, including tissue receipt and storage, solution preparation, probe preparation, colorimetric ISH, and equipment and other laboratory maintenance functions are governed by Allen Institute Standard Operating Procedures (SOPs) with revision control.



Figure 1. Workflow diagram for generation of gene expression data in human cortex. The workflow includes coordination of available tissue and probes, tissue sectioning, tissue assessment, ISH, image acquisition, image QC, informatics processing for expression detection and measurement and annotation QC. Ultimately all images that pass QC become available for public access online.

Gene Selection

The process of gene selection began with identification of four major categories constituting thematically interesting datasets for a broad user community. These categories are:

- Cortical cell-type markers. These markers were identified through mining of the ABA Mouse Brain, have unique or specific laminar or regional patterns of expression in mouse cortex, and include interneuron, glial and vascular markers (for examples, see Supplemental Data 5 and Supplementary Table 1 in reference 1).
- Gene families important for neural function. These families include ion channels, G-proteincoupled receptors (GPCRs), transporters, synaptic proteins, membrane proteins, and peptide or protein ligands. Particular emphasis is placed on ion channels and genes related to GABAergic neurotransmission.
- 3. Disease-related genes. Genes were selected by searching through available literature to identify genes (i) conferring disease susceptibility, (ii) known to be involved in physiological pathways implicated in disease, or (iii) encoding known drug targets. The diseases include autism, schizophrenia, epilepsy, microcephaly, neurodegenerative diseases, depression and anxiety and intellectual and developmental disabilities.
- 4. Comparative genomics. This category comprises genes identified in the literature as showing accelerated evolution, being under positive selection, or showing microarray-based gene expression differences either between rodents and primates or between non-human and human primates. Genes with human lineage specific amplifications compared to non-human primates are also included.

As one might expect, there is considerable overlap of genes among these four categories and genes that appear in more than one category receive higher priority for data generation.

Postmortem Human Tissue

Subjects

Frozen postmortem tissue samples from adult male and female subjects at least 20 years of age were obtained from the brain tissue collection of the Neuropathology Section of the Clinical Disorders Branch, GCAP, IRP, National Institute of Mental Health, NIH, Bethesda, MD. Specimens were collected, processed and characterized as previously described (2). Briefly, after obtaining informed consent from legal next-of-kin, demographic information and medical, substance use and psychiatric history was gathered. A postmortem clinical diagnosis was independently established by board-certified psychiatrists based on family interviews consisting of the Structured Clinical Interview for DSM-IV – clinician version (SCID-CV), the NIMH psychological autopsy interview and the Diagnostic Evaluation After Death of psychiatric, medical, police, and autopsy reports (3). Cases and specimens were further characterized for history of cigarette smoking, toxicology for illicit substances and prescription medications, a macro- and microscopic neuropathological examination by a board-certified neuropathologist, manner of death, verification of age at death and postmortem interval (PMI). PMI was calculated as time elapsed, in hours, between pronounced time of death and time of tissue freezing. For cases in which time of death was not pronounced by hospital or emergency medical personnel, time of death was estimated as the midpoint between the time the individual was last seen alive and the time the decedent was discovered.

Subjects selected for this study had normal neuropathological examination results and no known history of neuropsychiatric disease, as determined by the above screening mechanism. Evidence of drug use, as determined from interviews or by toxicology tests, led to exclusion of cases from this cohort. Cases in which manner of death was suicide or in which death was due to drug overdose or poisoning were also excluded.

Tissue Retrieval and Processing

As previously described (2), brains were removed from the skull and kept cold on wet ice until frozen. The cerebellum and brainstem were removed and sectioned in the horizontal plane. The forebrain was hemisected and cut into coronal slabs approximately 1 to 1.5 cm in thickness. Each section or slab was then rapidly frozen in a bath of dry ice and isopentane and stored at -80°C until removed for excision of visual cortex and middle temporal cortex.

Visual cortex is excised from frozen slabs, using the calcarine fissure as the primary landmark. Visual cortical samples contain Brodmann's areas 17 and 18, taken from the occipital pole and the slab just rostral to the occipital pole. The more rostral samples are preferentially used for clearer representation of cortical layers. Temporal cortex is also obtained from each subject, with dissection occurring at the level of the midbody of the hippocampus. The temporal cortical samples generally comprise Brodmann's areas 21, 22 and, in some cases, 20. Most of the samples are obtained from left cerebral hemisphere and are approximately 1.8 cm x 4 cm in order to fit on a standard microscope slide. See Figures 2A and 2B for general representations of the regions of interest.

One frequently used indicator of tissue quality is brain tissue pH, suggested to be an objective measure of agonal state (4) which in turn has been reported to affect RNA quality (5; 6). Tissue pH has been directly correlated with RNA quality (2; 6; 7) and appears to be stable after death (i.e. is not affected by PMI) and during freezer storage (8). Interestingly, it has been reported that not all mRNAs are equally sensitive to pH differences (8; 9). Although much of the research on pH and RNA quality has been focused on microarray analyses of postmortem tissue, gene expression detection using ISH in postmortem tissue is also likely subject to the same considerations. Cerebellar pH is determined for all tissue samples in this study as described (2) and any samples found to have pH < 6 are excluded from this study. Other tissue criteria include restriction of PMI generally to less than 36 hours, but longer PMI does not exclude



Figure 2. Schematic representations of visual cortex (A) and temporal cortex (B) regions excised and characterized for gene expression using colorimetric ISH. Cortical region reference images were created for the Allen Institute by Dr. Jacopo Annese, The Brain Observatory, University of California, San Diego.

Tissue Receipt

Upon receipt, tissue is photodocumented and information is entered into LIMS for future tracking throughout the data pipeline. Tissue is stored in liquid nitrogen until removed for validation or sectioning.

Regulatory Compliance

A protocol describing the project to characterize gene expression in postmortem human brain was submitted by the Allen Institute and reviewed by Western Institutional Review Board's Regulatory Affairs Department, resulting in issuance of a Determination of Exemption.

Tissue Validation

Prior to sectioning for ISH, all tissue samples are tested for (a) confirmation of region of interest based on expected cytoarchitecture, (b) RNA quality, (c) absence of senile plaques and (d) absence of severe ice crystals. Tissue samples that fail these criteria are not used in the study.

Confirmation of Region of Interest

Two sample sections are taken for Nissl staining to confirm region of interest based on expected cytoarchitectural features. A thionin-based Nissl staining protocol used for the ABA – Mouse Brain (1; Supplemental Methods 1) was modified to increase staining intensity for human tissue by increasing incubation time in thionin stain and decreasing the duration of water washes and ethanol dehydration steps. All other steps are as previously described.

RNA Quality

For RNA quality assessment purposes, two tissue sample sections weighing between 2 and 5 mg and no more than 10 mg each are used for RNA extraction. RNA is extracted using the MELT[™] Total Nucleic Acid Isolation system (Ambion, Foster City, CA), per the manufacturer's protocol. The MELT extraction method uses an enzymatic homogenization and lysis process that inactivates RNases. Samples are stored at room temperature for up to 1 week after lysis and are processed in 96-well plates to enable high-throughput processing. The MELT extraction process captures nucleic acids using magnetic beads. RNA is eluted with 20 µl nuclease-free water and stored at -80°C. Yields are typically between 300-500 ng total RNA. No further purification of RNA samples is performed.

RNA quality is determined for each specimen using high-resolution capillary electrophoresis on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, California) and Agilent's RIN software algorithm to generate RNA Integrity Numbers. RNA is eluted in 20 μ l nuclease-free water, standardized to a concentration of 5 ng/ μ l and 1.0 μ l is run on a Pico Bioanalyzer chip. RNA integrity numbers are based on the entire electrophoretic trace and range from 1 to 10, where 1 corresponds to completely degraded RNA and 10 corresponds to perfectly intact RNA (12). RIN information for each specimen is provided in the online viewer, under specimen details.

It is important to note that RIN values vary due to sample specific characteristics and are also influenced by assay parameters for RNA extraction and RIN measurement. The extraction methods used here were chosen to (i) accommodate small tissue amounts, (ii) provide a safer enzymatic-based method of homogenization for working with human tissue and (iii) enable high-throughput analysis, which is required for this project.

Detection of Ice Crystals and Senile Plaques

All tissue samples are assessed for senile plaques by Thioflavin S staining and for ice crystals by ahaematoxylin and eosin (H&E) staining. The presence of either plaques or ubiquitously distributed large ice crystals is grounds for failing tissue for further use in ISH studies. Thioflavin S staining protocols are based on previously described methods (10). Slides that have been fixed, acetylated and dehydrated as described below are stained for 7 minutes with a mixture of Thioflavin S (Sigma; 0.125 mg/mL) and Sytox Orange (Invitrogen; 0.1 μ /mL) in 50% EtOH. They are then rinsed in fresh 50% ethanol and water then coverslipped using Hydromatrix and viewed using a fluorescence microscope to visualize stained plaques (Thioflavin S) and cell nuclei and neurites (Sytox Orange).

To assess ice crystal presence and severity, a representative section from each tissue sample is subjected to a regressive H&E stain (11). After sectioning, tissue is either fixed with neutral buffered formaldehyde or fixed, acetylated and dehydrated as described below. Slides are processed either manually or with autostainers depending on the required throughput. Sections are stained first with commercially prepared Harris haematoxylin, differentiated in 1% HCl in 70% ethanol, blued with 1% lithium carbonate and stained in 1% eosin Y in 1% aqueous calcium chloride. Sections are then dehydrated in a graded series of 70%, 95% and 100% ethanol, cleared in xylene and coverslipped with either DPX or CureMount mounting media.

Sectioning

Frozen tissue samples are sectioned in Leica CM3050 S cryostats (object temperature, -10°C; chamber temperature, -15°C) at 20 µm thickness in the coronal plane from anterior to posterior. One section is placed on each positively charged Superfrost Plus[™] 1" x 3" microscope slide (Erie Scientific Co, Portsmouth, NH), pre-printed with a unique identifying barcode for tracking. Specimen numbers are also printed onto the slides for tracking purposes. Samples too large to fit on slides are trimmed prior to sectioning. Slides are organized into groups of 4, representing 4 tissue sections spaced 1 mm apart across the sample. Each group of 4 slides constitutes a series; tissue samples typically yield two sets of 50 series depending on the thickness of the sample. For anatomical and cytoarchitectural reference, two of the 50 sectioning series (series 25 and series 50) are designated for Nissl staining so that a Nissl reference is available every 500 µm throughout the tissue block. A third series is used for positive control genes and a negative control. Each of the remaining series is hybridized with a single gene probe.

Following sectioning, slides designated for ISH are allowed to air dry and tissue is fixed, acetylated and dehydrated according to standard protocols as described (1; Supplemental Methods 1). Briefly, tissue is fixed for 20 minutes in 4% neutral buffered paraformaldehyde (PFA)and rinsed in 1x PBS, acetylated for 10 minutes in 0.1M triethanolamine with 0.25% acetic anhydride, and subsequently dehydrated using a graded series of 50%, 70%, 95% and 100% ethanol. Slides that pass section quality checks are stored at room temperature in Parafilm[™]-sealed slides boxes until used.

Probe Design and Synthesis

For labeling target mRNA in tissue sections using ISH, digoxigenin-labeled riboprobes are designed and synthesized according to specific criteria. In general, the design and synthesis process follows previously described methods used to generate probes for the ABA – Mouse Brain (1; Supplemental Methods 1) with some modification. Briefly, using sequences obtained from RefSeq and a semi-automated process based on Primer3 software (13), probes are designed to be between 400-1000 bases in length (optimally > 600 bases) and to contain no more than 200 bp with > 90% homology to non-target transcripts. In addition, to allow comparability of mouse and human gene expression datasets, human probes are designed to have > 50% overlap with the existing ABA – Mouse Brain probe when the mouse and human genes are orthologous. Riboprobes are synthesized using standard *in vitro* transcription (IVT) reactions based on PCR templates prepared from human cDNA clones (NIH Mammalian Gene Collection, Open Biosystems, Huntsville, AL) or pooled cDNA synthesized from human brain total RNA. cDNA was prepared from human brain RNA from prefrontal, temporal, parietal, occipital, and frontal cortical areas as well as medulla and cerebellum (Ambion, Austin, TX) using Superscript III RTS First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA), then pooled in equal amounts to provide templates for PCR.

PCR primers are obtained from Integrated DNA Technologies (Coralville, IA) at a final concentration of 10 μ M, and designed with GC content between 42% – 62 % and an optimal size of 22nt with lower and upper limits of 18 and 26 nt, respectively. For cDNA clones, the clone sequence is compared with RefSeq sequences, and consensus sequences with > 98% homology across 80% of the total length are used to develop probes. When a clone is used as a template, a single PCR is used requiring only a forward and reverse primer with an additional SP6 RNA polymerase binding sequence (GCGATTTAGGTGACACTATAG). When using brain cDNA as a template, probes are generated against sequences within a region 3000 bp from the 3' end using 3 primers: forward, reverse, and a nested reverse primer containing the SP6 RNA polymerase binding sequence. cDNA primers undergo a BLAST analysis to verify that only target sequence will be amplified. All cDNA reactions are run on the Bioanalyzer for quality control and all PCR products generated from cDNA template are sequenced from both ends using MegaBACE and ABI3700 capillary instruments at the University of Washington High Throughput Genomics Unit.

Standard conditions for PCR and IVT reactions are as described (1; Supplemental Methods 1). IVT reactions are diluted to working stocks of 30ng/µl with THE (0.1mM Sodium Citrate pH 6.4, Ambion). Aliquots are stored in one- or two-use volumes to minimize freeze/thaw cycles. IVT dilutions are stored at -80°C. For hybridization, the probe is diluted 1:100 (to 300ng/ml) into *in situ* hybridization buffer (Ambion) in 96-well ISH Probe Plates. Each well provides probe for one ISH slide. Probe plates are stored at -20°C until used in an ISH run

All PCR and IVT products are run on the Bioanalyzer for size and morphology quality control. Specifically, PCR products that are not of the correct size (+/- 100bp) or that show multiple products are not used to generate riboprobes. IVT products that are shorter than their predicted size are not used. It is common to see IVT products that run slightly larger than their predicted molecular weight, or as multiple peaks, due to secondary structure of the RNA. IVT products with multiple bands are not used for ISH unless the additional bands are determined to result from secondary structure.

Colorimetric In Situ Hybridization

A colorimetric, digoxigenin-based method for labeling target mRNA is used to detect gene expression on human tissue sections. Detailed descriptions of the high-throughput platform, protocols, and reagent preparation are available elsewhere (1, Supplemental Methods 1). In general, slides containing tissue sections are placed in flow-through chambers on temperature-controlled racks on computer-controlled Tecan Genesis liquid handling platforms for addition of solutions. The protocol begins with steps to block

endogenous peroxidase activity and permeabilize the tissue, followed by subsequent hybridization of digoxigenin-labeled probes to target mRNA. Following a series of washes to eliminate excess probe, the remaining bound probe is subjected to a series of enzymatic reaction steps to detect and amplify digoxigenin signal. First, a horseradish peroxidase (HRP)-conjugated anti-digoxigenin antibody is added, followed by biotin-coupled tyramide that is converted by HRP to an intermediate that binds to cell-associated proteins at or near the HRP-linked probe. Neutravidin conjugated with alkaline phosphatase (AP) is then bound to biotin and BCIP/NBT is added. A blue/purple particulate precipitate forms as a result of the enzymatic cleavage of BCIP by AP and subsequent indole reaction with NBT. Finally, the colorimetric reaction is stopped by washing with EDTA and fixed with 4% PFA. This entire process occurs over the course of approximately 23.5 hours on the Tecan automated platform.

Each ISH run contains several positive controls: human GAP43 and CTNND2 are included as robust and moderately expressed gene controls, respectively. Human PVALB was originally included as a control gene but was discontinued because it was less informative as a control gene than initially anticipated. Because it is often the case that gene expression results for human probes are simply not yet known, a well-characterized Drd1a positive control in mouse tissue is used to provide verification of a successful ISH run. A negative control (no probe) slide is also included as an indication of background for each ISH run. In addition to these positive and negative controls, PCP4 and CART are included as regional indicators for visual cortex (VC) and temporal cortex (TC), respectively. The PCP4 expression pattern delineates the boundary of Brodmann's area 17 and Brodmann's area 18. CART was selected for detection of Brodmann's area 21 boundaries in TC based on prior literature (14) but validation of this gene as a marker for this region has been made difficult by the subtle cytoarchitectural boundaries between Brodmanns' areas 21, 22 and 20.

To reduce background signal that interferes with signal from target mRNA, an acid alcohol wash step is performed after completion of the hybridization process. Slides are rinsed 4 times (1 minute each) in acid alcohol (70%, adjusted to pH = 2.1 with 12N HCl) and rinsed 4 times in milliQ water (1 minute each). Acid alcohol and water solutions are refreshed every fourth rack to ensure that all slides are rinsed in clean solution.

Image Acquisition

Two image acquisition platforms have been used for scanning and digitizing human cortex data. Initially, images were generated using a previously described (15) high-throughput automated microscopy platform developed at the Allen Institute for Brain Science. Each stand-alone image capture system (ICS) unit consists of a Leica DM6000B automated brightfield microscope, Leica DC500 camera, Ludl BioPrecision stage with automated slide loader, Microscan CCD-3 barcode imager, and an HP wx6200 dual-processor workstation. Custom ScopeController software combined with ImagePro Plus commercial software handle all system integration, configuration and image acquisition processes. Following transfer from 25-slide cassette holders to a microscope stage, each slide is barcode scanned for data tracking purposes, and a color, white balance and background correction check is performed. For image acquisition, each slide is initially pre-scanned with a 1.25x objective and tissue boundaries are defined. The slide is subsequently scanned using a 10x objective for higher resolution. Five autofocus points are required to maintain good focus across the human tissue section. Four of these points are located at the top, bottom, left and right edges of the tissue border determined by a tissue masking algorithm. The fifth point is located at the mathematical center of the four edge points. Image resolution of this system is 1.05 µm per pixel.

Currently, image acquisition is completed using ScanScope® scanners (Aperio Technologies, Inc; Vista, CA). These instruments use a line scan camera that is continually adjusted for focus based on a variable number of focus points. This is an advantage for large human tissue sections that tend to have more variation in height. The Aperio system increases the speed of image acquisition approximately 5-fold, primarily because of increased speed of the autofocus algorithm. The ScanScope scanner uses a 20x

objective that is downsampled in software to minimize the data volume that is acquired for this project. This downsampling provides similar image resolution (1.00 µm/pixel) to the ICS scanning systems.

Data Processing

Once images have been acquired, the Informatics Data Pipeline (IDP) manages image preprocessing, image QC, ISH expression detection and measurement, Nissl processing, annotation QC and public display of information via the Web application. The IDP has been described in detail previously (16), and has been modified slightly for processing human cortical images.

IDP Cluster Computation Requirements

One major challenge for the processing pipeline is the large image size that results from a human tissue section. To support the processing of human cortex tissue images generated by the ICS or by the ScanScope, the informatics processing platform was migrated to a 64-bit Linux platform, including cluster hardware, system software and IDP applications. Cluster blades are configured to operate in 64-bit mode with at least 8GB of main memory each to provide dedicated blades with 8 - 14GB of working memory to execute the processing modules.

IDP Processing Modules

Three modules constitute the processing pipeline for human cortex images: image preprocessing, ISH expression detection and NissI processing. In image preprocessing, scanned ISH and NissI images are converted and processed to provide more consistent white background intensities and orientation across samples. The preprocessing steps differ slightly for images acquired using the ICS vs. the Aperio system. Specifically, ICS images are first stitched from tiles in Tiff format, white balanced and finally converted to JPEG 2000 file format. Aperio images are first converted to JPEG 2000 format, then orientation adjusted and white balanced. In either case, the final products are images in JPEG2000 compressed format for further pipeline processing and analysis.

For ISH expression detection, adaptive image processing techniques are applied to 10x full-resolution ISH images to detect and quantify gene expression. As an example of the scale of the engineering challenge, the number of detected expressors (cells) can reach the level of nearly 2 million in a typical 3 GB cortex image. With large tissue sizes, image quality with regard to uniformity is compromised and robust detection becomes much more dependent on stain intensity, contrast and focus. An algorithm based on techniques used for the ABA – Mouse Brain (1; Supplemental Methods 2) was significantly redesigned to accommodate the long addressing mode and full image resolution needs that result from large image sizes. The resulting module produces a mask of detected expressor objects and a set of numerical values describing the statistical attributes of gene expression. The mask image with measured intensity of expression is then pseudo-color coded and converted to AFF file format for Web display.

Nissl stained sections are processed to determine tissue area size and estimate grey matter area as a reference for computation of the relative expressing density indicator available in the Web application when viewing images. Computed expression measurements are normalized by estimated tissue area from the closest Nissl section to provide a relative indication of expression within the whole tissue or gray matter area (see Figure 3).

Quality Control and Public Display

After image acquisition, and during the course of data processing, two additional quality control steps occur. Once image preprocessing is complete, images become available in LIMS for image quality control to ensure focus criteria are met and to provide an initial indication of the presence of signal. If focus criteria are not met, the images are 'failed' and the appropriate slides are rescanned. If focus criteria are met, the images are passed and proceed through the IDP for ISH expression detection.

When ISH expression detection is complete, a final quality control check is performed prior to public release of images. This final QC primarily verifies anatomic location of each tissue sample based on

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Nissl stained sections and marker gene data. Primary visual cortex (Brodmann's area 17) is differentiated from surrounding cortical areas in Nissl stained sections by the separation of layer 4 into 4a, b and c. This separation is distinguishable by the Line of Gennari, a prominent band of highly myelinated fibers in cortical layer 4c. This area is confirmed by the marker gene PCP4, which marks layer 4c in primary visual cortex. The adjacent secondary visual cortex (Brodmann's area 18) is easily identifiable once Brodmann's area 17 is established. Identification of temporal cortex is aided in Nissl stained sections by identifying small stacks of large pyramidal cells (~4-6 cells stacked) in layers 3 and 5 of Brodmann's area 21, disorganization of layer 4 and lacunar distribution (appearing as holes or gaps in the cellular organization) of cells in layer 3. These features can be seen in comparison to the clear columns of cells that span through all cortical layers in Brodmann's area 22, and the more organized and denser grouping of granular cells in layer 4 of this area.

Images that meet anatomic criteria are subsequently passed for public release. From time to time, slides may be rescanned to improve image quality. In these cases images in the database will be replaced with the most recent scan of the original slide.

For each set of gene images available in the online viewer, the closest set of Nissl stained sections can be accessed and viewed.



Figure 3: Segmentation and estimation of tissue and gray matter areas (not-yellow area) on a Nissl section.

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