Nutil: Neuroscience Image Processing and Analysis Utilities

Contents

Introduction

Nutil simplifies the pre-and-post processing of 2D brain image data from mouse and rat. Nutil is developed as a standalone application and requires no experience to execute. The user specifies the input and output folders and the parameters for the pre-and post- processing operations in the Nutil graphical user interface (GUI).

Pre-processing operations include conversion of images from JPEG, PNG and untiled TIFF to tiled TIFF format (*TiffCreator)*; 2D transformations of large tiled TIFF images (rotation, flipping and scaling) in addition to renaming, copying and downsizing (*Transform*); and resizing of JPEG, PNG and TIFF images with output in PNG format (*Resize*).

Post-processing (*Quantifier*) is used for the QUINT workflow. It enables the regional quantification of features such as cells in histological image series of rodent brain, with the regions defined by a reference atlas, such as the Allen Mouse Brain reference atlas or the Waxholm Space Atlas of the Sprague Dawley rat brain. All functions operate in batch, and operate in parallel on multiple CPUs.

PLEASE visit the EBRAINS page for information about the QUINT workflow, and to find tutorials, examples of use, demo videos and software download information:

https://ebrains.eu/service/quint

Nutil enables four operations detailed further in this user guide:

A. *TiffCreator***:** convert JPEG, PNG and normal TIFF images to tiled TIFF format
B. *Transform:* rename, rotate, resize and compile thumbnails of large tiled TIFF images
C. *Quantifier*: for the batch extraction, quantification and spatial analysis of labelling segmented from 2D mouse or rat brain section images (for example, immunohistochemical labelling).
D. *Resize:* for resizing JPEG/PNG images with output in PNG format.

Figure 1: Nutil GUI.

Installation and usage

Nutil is a stand-alone 64-bit Windows application. There are no installation procedures, just extract the folder and double click on "Nutil.cmd". Nutil is an integral part of the QUINT workflow developed by the Nesys laboratory.

Documentation: https://ebrains.eu/service/quint

Download: https://www.nitrc.org/projects/nutil/

Report issues: https://github.com/Neural-Systems-at-UIO/nutil

Slack workspace for informal discussion: quint-uiq9333.slack.com

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Also described in: Yates SC, Groeneboom NE, Coello C, Lichtenthaler SF, Kuhn P-H, Demuth H-U, Hartlage-Rübsamen M, Roßner S, Leergaard T, Kreshuk A, Puchades MA and Bjaalie JG (2019) **QUINT: Workflow for Quantification and Spatial Analysis of Features in Histological Images From Rodent Brain.** Front. Neuroinform. 13:75. doi: 10.3389/fninf.2019.00075.

Funding: This work was supported by the European Union's Horizon 2020 Framework Programme for Research and Innovation under the Specific Grant Agreement No. 720270 (Human Brain Project SGA1) and Specific Grant Agreement No. 785907 (Human Brain Project SGA2).
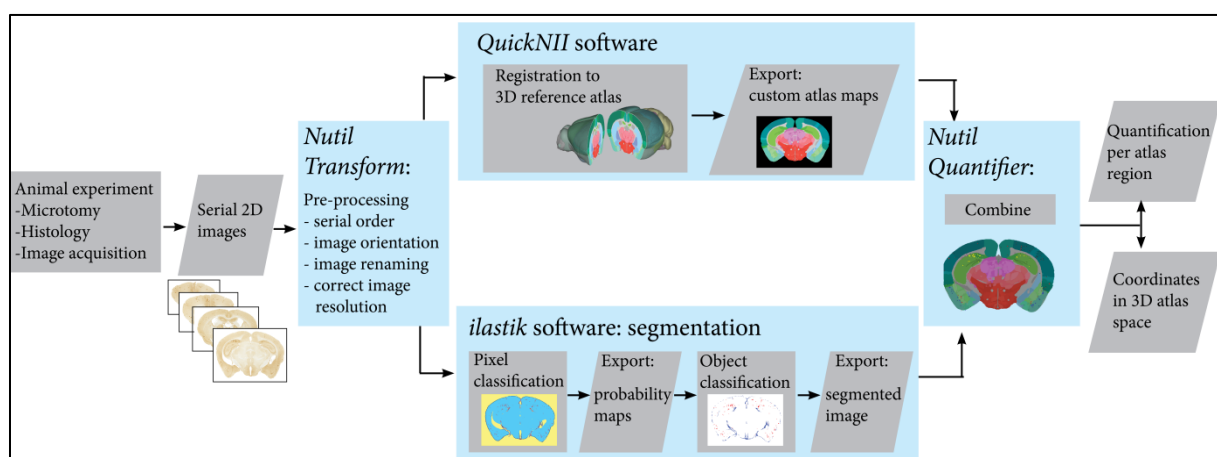

Figure 2: QUINT workflow

Operation: TiffCreator

TiffCreator converts JPEG, PNG, BMP, GIF and untiled TIFF images to the tiled TIFF format that is compatible with *Transform*.

TiffCreator operates in batch, converting all the images in an input folder and saving them in the specified output folder.
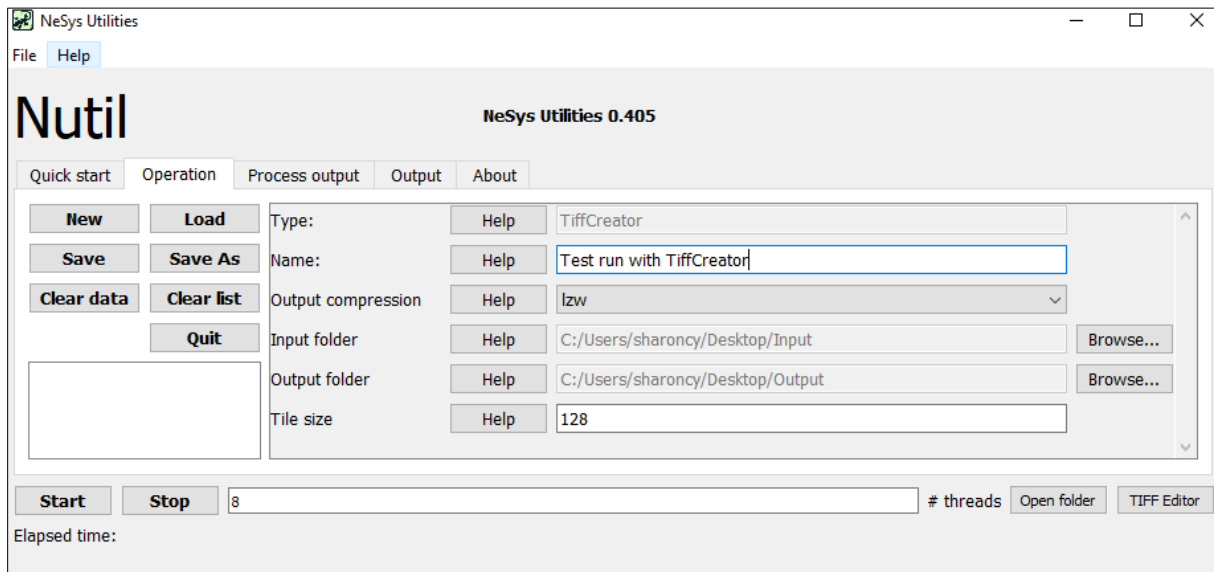

Figure 3: TiffCreator GUI

1. To begin, click "New". Select "TiffCreator".
2. Select the input folder (containing the images to be converted), output folder and enter the TiffCreator parameters in the Nutil GUI (e.g. output compression type).
3. Press "Save as". This saves a copy of the selected settings in a simple text file in .NUT format. The NUT file is for your own records but may be reloaded into Nutil via the "load" button.
4. *Nutil* automatically detects the number of core processor available (8 in the example). Choose one less than the total available to ensure adequate processing power (6 or 7 here) and press "Start". Wait until the process is complete.
5. The tiled TIFFs are saved in the specified output folder.

    Nutil includes an inbuilt user manual accessed via the "Help" buttons.

Operation: Transform

Transform allows the batch renaming, rotation, resizing and thumbnail compilation of tiled TIFF images (max size: 4 GB).

The tiled TIFF image format has an upper size limit of 4 GB. *Nutil* cannot transform or generate images that are bigger than 4 GB.
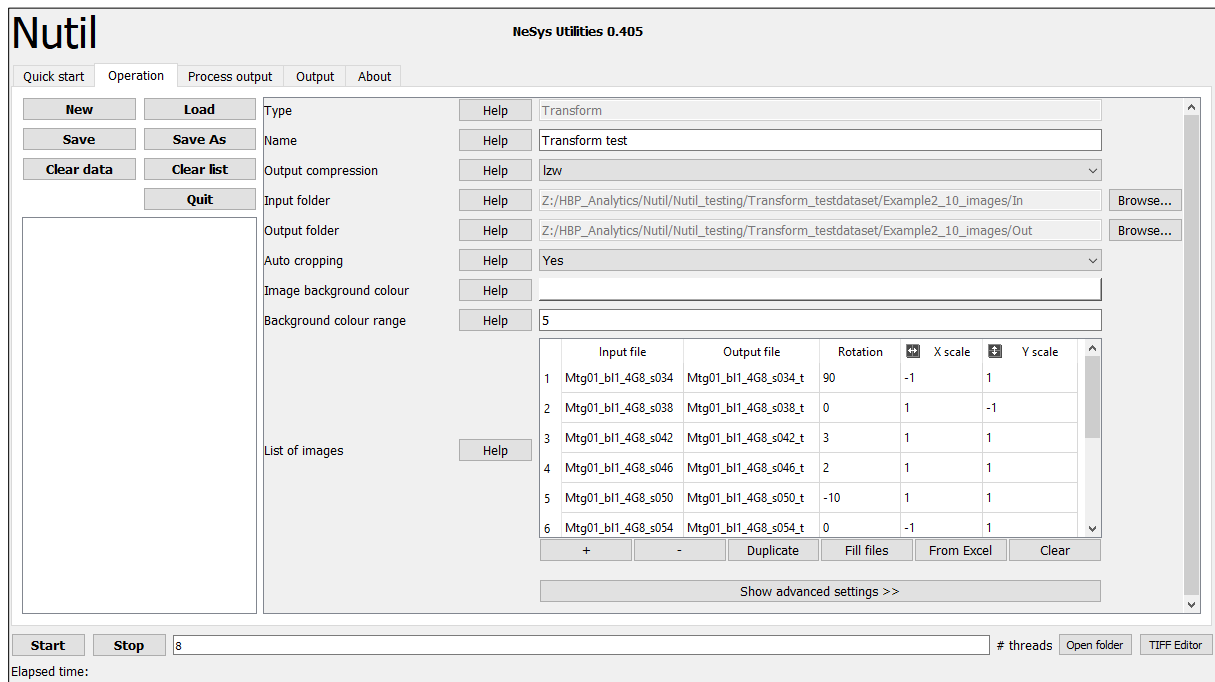

Figure 4: Transform GUI

1. Click "New". Select "Transform".
2. Select the input folder (containing the images to be transformed), output folder and enter the transformation parameters in the Nutil GUI. The GUI includes an inbuilt user manual accessed via the "Help" buttons.
3. Once everything is filled in press the "Save as" button. This saves a copy of the transformation parameters in a simple text file in .NUT format. This is for your own records, but may also be reloaded into Nutil via the "load" button.
4. *Nutil* automatically detects the number of core processor available (8 in the example). Choose one less than the total available to ensure adequate processing power (6 or 7 here).
5. Press "Start" and wait until the process is complete.

Operation: Resize

Resize enables rapid resizing of PNG, JPEG and untiled TIFF images by application of a resize factor or a fixed pixel width. The output images are in PNG format and are suited for ilastik segmentation.
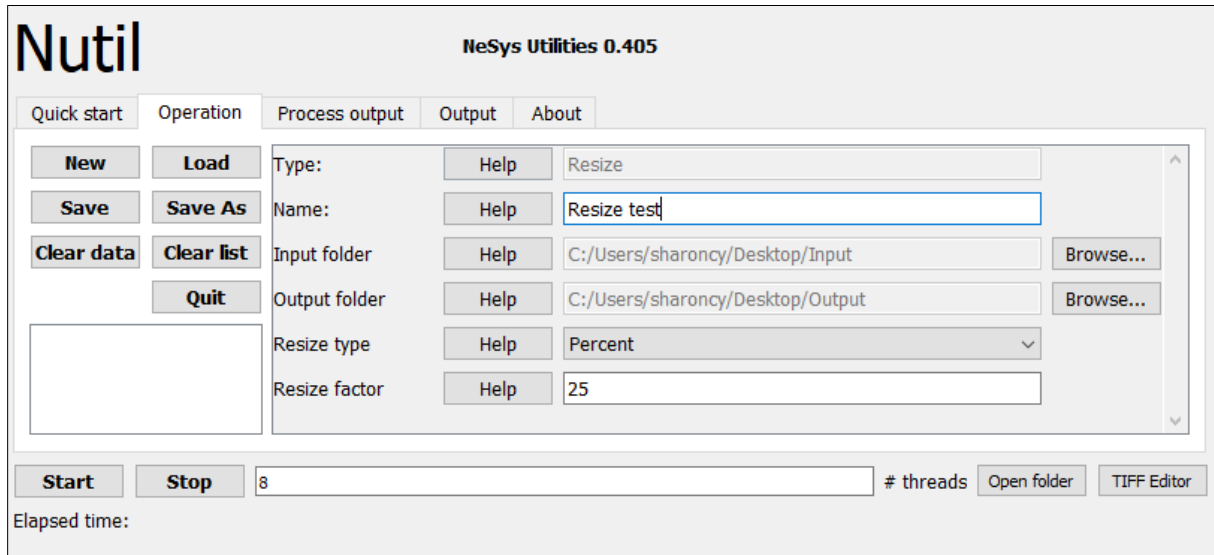

Figure 5: Resize GUI

1. To begin, click "New". Select "Resize".
2. Select the input folder, output folder and resize factor in the Nutil GUI (see the "help" buttons for more information).
3. Press "Save as". This saves a copy of the parameters in a simple text file in .NUT format. This is for your future records but may also be reloaded into Nutil via the "load" button.
4. *Nutil* automatically detects the number of core processor available (8 in the example). Choose one less than the total available to ensure adequate processing power (6 or 7 here) and press "Start". Wait until the process is complete.
5. The PNG files save automatically in the specified output folder.

Operation: Quantifier

Quantifier is an integral part of the QUINT workflow, and enables the regional quantification of features extracted from histological images, based on a reference atlas such as the Allen Mouse Brain Atlas (AMBA) or the Waxholm Atlas of the Spraque Dawley Rat.

File naming requirements

- The file names of the segmentation files and the brain atlas maps that correspond to a particular section image must contain the same unique ID.
- These unique IDs must also be present in the XML/JSON file containing the anchoring information. This happens automatically as long as the images that are anchored with *QuickNII* contain the unique IDs.

> **OBS!** The sXXX naming convention applies to both *QuickNII* and *Quantifier*. We therefore recommend changing the file names as the first step in the QUINT workflow. This can be done with *Transform (*see Figure 1).

- *Quantifier* support*s* IDs in the format: sXXX.., with XXX.. representing the section number. The section number should reflect the serial order and spacing of the sections (e.g. s002, s006, s010 for every 4th section starting with section 2). The IDs must match those in the XML or JSON file.

  Example: tg2345_MMSH_s001_segmentation.png
  (It is fine to include a string of letters and numbers followed by the unique ID).

  As *Quantifier* scans and detects the _s part of the name, the file name should not contain additional _s'. Example that would not work: tg2345_MMSH_ss_s001.png

- *Quantifier* also supports user-defined IDs using regular expressions – RegExp. For more information see the "help" button in the Nutil GUI.

Input files

1. Segmentations

Any image analysis software may be used to generate the segmentations. We use the Pixel and Object Classification workflows in the *ilastik* software (ilastik.org) with the Glasbey LUT applied with Fiji.
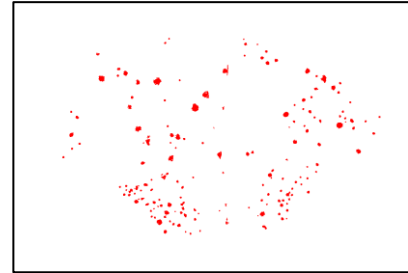
Requirement:

- Must be indexed 8-bit or 24-bit RGB images in PNG format.
- Must have the same proportions as the images <u>used to generate</u> the atlas maps. They do not need to be the same size as the images used to generate the atlas maps, and are typically larger in size.

   OBS! The segmentations will not have the same proportions as the actual atlas maps as QuickNII alters the proportions slightly.

- *Quantifier* is only able to extract one RGB (Red Green Blue) colour at a time. Apply one RBG colour to all of the objects of interest, and specify this colour code in the GUI (e.g. the objects in the segmentation here are red, which is RGB colour code: 255,0,0).

> *ilastik* is a versatile image analysis tool specifically designed for the classification, segmentation and analysis of biological images based on supervised machine learning algorithms.
>
> A user manual that describes how to use *ilastik* in the context of the QUINT workflow is included as part of the *Nutil* package. See: **ilastikUserManual.**
>
> *ilastik is available at:* http://ilastik.org/download.html

2. Brain atlas maps

The atlas maps are customised to match the cutting plane and proportions of the brain sections. They are generated with the *QuickNII* software (linear registration only) or with the *VisuAlign* software (if nonlinear refinements are required). They are in .FLAT format.

Visit and download here: https://ebrains.eu/service/quicknii-and-visualign

3. XML or JSON file containing the anchoring information.

Either the XML or JSON file from *QuickNII*, or the JSON file from *VisuAlign* may be used. They all contain the linear registration information that is needed to generate coordinate output. Nonlinear adjustment of the atlas maps with *VisuAlign* does not alter the linear coordinate information contained in the file.

QuickNII is a standalone software for affine spatial registration (anchoring) of section images - typically high resolution histological images - to a reference atlas such as the Allen Mouse Brain Atlas or the Waxholm Atlas of the Sprague Dawley Rat. Once all the sections are registered, *QuickNII* may be used to generate atlas maps that match the cutting plane and proportions of the experimental image data. The anchoring information (coordinates) is saved and stored in an XML or JSON file.

Theory: In *QuickNII*, the reference atlas is transformed to match anatomical landmarks in the experimental images. In this way, the spatial relationship between experimental image and atlas is defined, without introducing transformations in the original experimental images.

Method: The registration is user-guided with some automation. Following anchoring of a limited number of sections containing key landmarks, transformations are propagated across the entire series of images to reduce the manual work required. These propagations must be validated by visual inspection and typically require fine adjustments for most sections. A user manual that describes how to use *QuickNII* in the context of the QUINT workflow is included as part of the *Nutil* package. See: **QuickNII userguide.**

VisuAlign is a standalone software for applying nonlinear refinements (inplane) to an existing affine 2D-to-3D registration (the 2D-to-3D registration is performed with *QuickNII* and stored in the JSON file).

Theory: It is used to make manual adjustments to the atlas maps to better match the sections. The adjustments are nonlinear.

Method: Open the JSON file from *QuickNII* in *VisuAlign* and apply adjustments by simple drop and drag of markers placed on the image. The adjusted atlas maps may then be exported in .FLAT format and are compatible with *Quantifier*. *VisuAlign* does not update the linear coordinate information contained in the JSON file. A user manual that describes how to use *VisuAlign* in the context of the QUINT workflow is included as part of the *Nutil* package. See: **VisuAlign userguide.**

OBS! Make sure your XML or JSON file has anchoring information for every section image in your dataset.
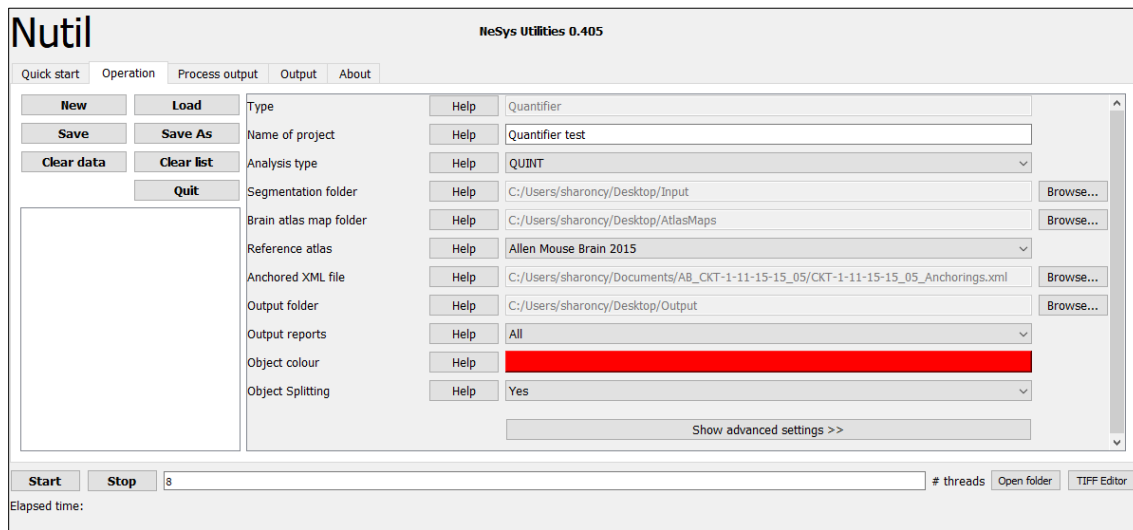
Running Quantifier

Figure 5: *Quantifier* GUI. Note the object colour has to match the colour of your segmented objects (here red colour).

1. Create three new folders: for example, titled "Segmentations", "Atlas_maps" and "Output". Transfer the segmentations to the segmentation folder, the atlas maps to the atlas map folder. Leave the output folder empty.
2. To begin, click "New". Enter a name for your project.
3. Press the "browse" buttons and navigate to the folders containing the segmentations, the atlas maps and the output folder, and to the XML or JSON file containing the anchoring information.
4. Select the reference brain atlas. **This must match the version which was used to generate the atlas maps** (Allen mouse brain 2015 or 2017, or Waxholm Space Atlas of the Sprague Dawley Rat v2 or v3).
5. Fill in the rest of the form. The software includes an inbuilt user manual accessible via the "Help" buttons, and gives more information for each parameter. Some of these are described in more detail in the section below.
6. The "show advanced settings" button reveals more settings that may be altered (e.g. min object size cut-off, option to generate customised reports, etc). This gives flexibility for customised analysis. If nothing is changed in the advanced settings, the default settings shown below are applied.

Advanced Parameter	Default settings
Minimum object size	1 pixel
Pixel scale	1 pixel
Use custom masks	No
Output report type	CSV
Apply customised regions	Default
Coordinate extraction	All (Yes, for whole series and per section)
Pixel density	1 coordinate per pixel
Nifti size	0 (no nifti file generated)
Unique ID format	_sXXX…

7. Press "Save as". This saves a copy of the settings in a simple text file in .NUT format. This is useful for future reference, and may be reloaded into *Nutil* via the "load" button (for example, to repeat the analysis on a new set of images).
8. *Nutil* automatically detects the number of core processor (threads) available on the computer (8 in the example). To ensure adequate processing power, choose one less than the total available (6 or 7 here) and press "Start". Wait until the process is complete.
9. The output files are automatically saved in the specified output folder.

Figure 6: Quantifier advanced settings GUI

Quantifier settings explained

Nutil has "help" buttons throughout with information on each parameter.

Some of the Quantifier settings are described in more detail below:

Object splitting

In Quantifier, users must specify whether to turn on or off "object splitting". Object splitting divides segmented objects that overlap atlas regions, with individual pixels assigned their precise location. This gives accurate load measurements (load is the percentage of the region occupied by objects), but invalidates the object counts.

Recommendation:
Select <u>NO</u> for small objects to get accurate object counts (e.g. cells).
Select <u>YES</u> for large objects that overlap atlas regions (e.g. connectivity data). This gives precise load output. See the help button for more information.

Custom masks

The mask feature is optional. It allows the application of masks to define which parts of the sections to <u>include</u> in the analysis. The mask is applied in addition to, and not instead of, the reference atlas maps. This is particularly useful for investigating expression differences in the right and left hemisphere, as a mask can be applied to differentiate the two sides.

- To use the mask feature, select "yes". This brings up a "custom mask folder" and "Custom mask colour" option.
- Create binary masks (black and white) in PNG format with an application such as *NIH ImageJ*, *Adobe Photoshop* or *GIMP*. These should have the same proportions as the segmentations (but not necessarily the same size).
- Name these with the unique ID for the section and a "_mask" extension. File name example: Bxb_hgt_s002_mask
- Navigate to this folder containing the masks.
- Click on the field for the "Custom mask colour". Select the colour in the mask that corresponds to the ROI to <u>include</u> in the analysis. For example, for an analysis of the left hand side of an image with the mask shown here, specify black (RGB code: 0,0,0).
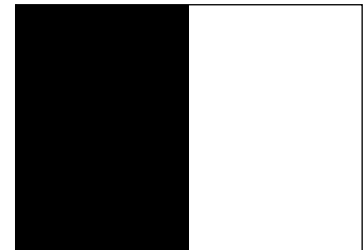
Customised reports

Quantifier generates two or three sets of reports:

- *RefAtlasRegion* reports contain quantifications per atlas region based on the finest level of granularity of the atlas.
- *CustomRegion* reports contain quantifications for broader regions, such as cortex and hippocampus ("default"), or user defined regions ("custom").
- *Object* reports contain information about individual objects and are only generated with object splitting switched OFF.

The custom regions are compilations of reference atlas regions. Users have the option to either define their own using the CustomRegionsTemplate.xlsx, or to use the default regions included in the *Nutil* software. More information on the default regions are found in the CustomRegion files in the *Nutil* package (see folder titled "CustomRegion" and navigate to the xlsx file that corresponds to your atlas). The "default" option is a whole brain analysis. It includes all the reference atlas regions subdivided into broad regions.
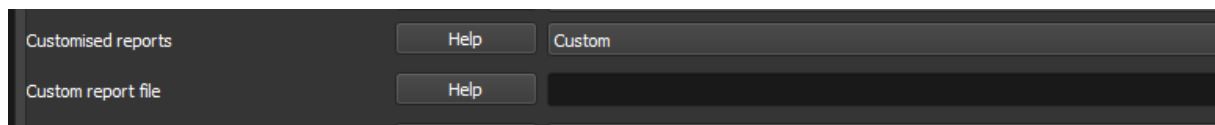
How to define your own regions

1. To define your own regions, use the *CustomRegionsTemplate.xlsx* that is included in the Nutil package, and populate as described below:

	A	B	C	D
1	**Custom brain region**	Cortex	Fibretracts	Hippo
2	**RGB colour**	255;0;0	255;0;255	255;255;0
3	**Colour**	red	fusia	yellow
4	**Atlas Ids**	8	1009	1089
5		567	967	1080
6		688	885	375
7		695	949	382
8		315	840	391
9		184	1016	399
10		68	21	407
11		667	665	415
12		500	538	423
13		107	459	431
14		219	900	438
15		299	848	446
16		644	876	454
17		947	916	463
18		985	336	471
19		320	117	479

ROW 1: assign your own names to the regions (e.g. Cortex).

ROW 2: assign colours to the regions. Do this by typing a RGB colour code in the following format: 255;0;0 (for red). This colour will be assigned to the objects located in the custom region for the purposes of the image and coordinate output (for display purposes only).

ROW 3: enter the colour name (this is for your information only).

ROW 4: define the region by listing the reference atlas IDs that you wish to include. The excel sheets in the AtlasHierarchy folder list all the regions and IDs for each atlas.

For mouse, see the *ABAHier2015.xlsx or ABAHier2017.xlsx* file for the full list of regions and IDs.

For rat, see the *WHS_rat_atlas_v2.xlsx or WHS_rat_atlas_v3.xlsx* file for the full list of regions and IDs.

NOTE: The default .xlsx may be used as a guide for filling out the template.

How to interpret the output

1. **Reports** (CSV or HTML format)

Region ID	Region Name	Region pix	Region are	Area unit	Object cou	Object pix	Object are	Area unit	Load
0	Clear Label	0	0	um2	207	10028	20056	um2	0
997	root	85.765518	171.53103	um2	0	0	0	um2	0
8	Basic cell groups and regions	0	0	um2	0	0	0	um2	0
567	Cerebrum	0	0	um2	0	0	0	um2	0
688	Cerebral cortex	0	0	um2	0	0	0	um2	0
695	Cortical plate	0	0	um2	0	0	0	um2	0
315	Isocortex	0	0	um2	0	0	0	um2	0
184	Frontal pole, cerebral cortex	0	0	um2	0	0	0	um2	0
68	Frontal pole, layer 1	0	0	um2	0	0	0	um2	0
667	Frontal pole, layer 2/3	0	0	um2	0	0	0	um2	0
500	Somatomotor areas	0	0	um2	0	0	0	um2	0
107	Somatomotor areas, Layer 1	0	0	um2	0	0	0	um2	0
219	Somatomotor areas, Layer 2/3	0	0	um2	0	0	0	um2	0
299	Somatomotor areas, Layer 5	0	0	um2	0	0	0	um2	0
644	Somatomotor areas, Layer 6a	0	0	um2	0	0	0	um2	0
947	Somatomotor areas, Layer 6b	0	0	um2	0	0	0	um2	0
985	Primary motor area	85239.234	170478.46	um2	33	1767	3534	um2	0.020730
320	Primary motor area, Layer 1	345318.34	690636.68	um2	32	1871	3742	um2	0.005418

RefAtlasRegions

Report with output organised based on all the regions in the reference atlas: per section and for the whole series (all sections combined).

> **IMPORTANT**: The *Allen Mouse Brain Reference Atlas* includes regions that are not actually delineated in the atlas. These regions are either big regions that have been delineated into smaller regions and so are not assigned to any pixels in the reference atlas, or are smaller regions that are not delineated. In the reports, these regions have no results (zero for region pixels and for object pixels) and should be excluded from analysis.
>
> The ***Clear Label ID*** covers objects that fall outside of the atlas maps.

CustomRegions

Reports with the output organised based on the customised regions defined in the CustomRegionsTemplate.xlsx: per section and for the whole series.

Objects

List of all the objects in the whole series and per section. By switching "ON" the "display object IDs in image file and reports" feature, a unique ID is assigned to each object in your dataset. These IDs are then displayed in the image files and in the object reports.

In each report, interpret the results as follows:

|	
Region pixels	No. of pixels representing the region.
Region area	Area representing the region
Area unit	Region area unit
Object count	No. of objects located in the region. NOTE: Object counts are not generated if object splitting is switched "on".
Object pixels	No. of pixels representing objects in this region.
Object area	Area representing objects in this region.
Load	Ratio of Object pixels to Region pixels (Object pixels/Region pixels).

2. IMAGES

- Segmentations superimposed on the atlas maps in PNG format.
- The object colours are assigned based on the customised regions. If no regions are specified, or object falls outside of the specified areas, the objects are shown in red by default.
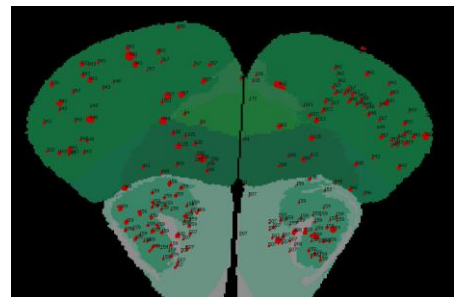
3. COORDINATES

JSON files containing point clouds that can be visualised with the *MeshView Atlas Viewer*.

The viewer is available via the MediaWiki link here: www.nitrc.org/projects/meshview
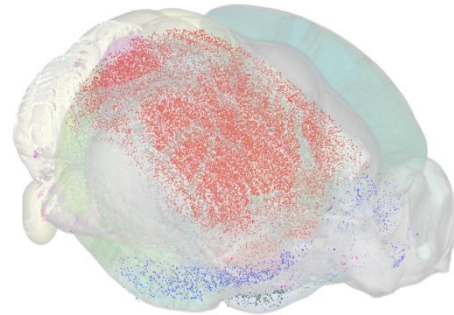
4. NUT file

The NUT file is a text file containing the analysis settings. This can be loaded into Nutil Quantifier with the "load" button.

To view, open with *Notepad.*


```
02_12_19.nut - Notepad

File   Edit   Format   View   Help

type = Quantifier
name =
analysis_type = QuickNII
quantifier_input_dir = Z:/HBP_Analytic
quantifier_atlas_dir = Z:/HBP_Analytic
label_file = Allen Mouse Brain 2015
xml_anchor_file = Z:/HBP_Analytics/Nut
quantifier_output_dir = Z:/HBP_Analyti
output_report = All
extraction_color = 255,0,0,255
object_splitting = No
object_min_size = 1
object_max_size = 100000
global_pixel_scale = 2
quantifier_pixel_scale_unit = um2
use_custom_masks = No
custom_mask_color = 255,255,255,255
output_report_type = XLSX
custom_region_type = Yes
custom_region_file =
```

Technical information

Development platform

Download: https://www.nitrc.org/projects/nutil/

Nutil is written as a stand-alone windows 64-bit application written in Qt C++, which enables the full usage of both memory and processor cores. *Nutil* can be downloaded and compiled from the github page. When performing batch processes, *Nutil* will utilise all cores available on the system.

The external libraries that are used in *Nutil* are:

- Libtiff for fast and efficient TIFF file handling (http://www.libtiff.org/)
- LibXLNT for excel file IO (https://github.com/tfussell/xlnt/)

TiffCreator

TiffCreator produces tiled TIFF files from JPEG, PNG and standard TIFF images, and employs the support of multiple CPUs for efficient, parallelised operations.

Transform

Transform enables rotations, scaling and thumbnail compilation of large tiff files (currently up to 4GB).

Quantifier

Quantifier identifies individual binary objects in a PNG file, while matching these to output from *QuickNII*. The method first finds and sorts areas by using a standard pixel filler routine. Afterwards, a random area pixel is chosen as the look-up in the binary *QuickNII* label slice for this particular image. When all areas have been assigned a label ID, multiple selections of pre-defined area IDs are assembled (ID list from the excel input file), and finally output reports are assembled and written to disk (in xlsl format). In addition, original ilastik .png files with colour/ID coding added to underlying atlas slice data are assembled and saved to the output *folder*.

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Cite

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Yates SC, Groeneboom NE, Coello C, Lichtenthaler SF, Kuhn P-H, Demuth H-U, Hartlage-Rübsamen M, Roßner S, Leergaard T, Kreshuk A, Puchades MA and Bjaalie JG (2019) QUINT: Workflow for Quantification and Spatial Analysis of Features in Histological Images From Rodent Brain. Front. Neuroinform. 13:75. doi: 10.3389/fninf.2019.00075.

Acknowledgements

Release notes

Can be found on https://www.nitrc.org/frs/?group_id=1365

Contact us

Report issues: https://github.com/Neural-Systems-at-UIO/nutil

Slack workspace for informal discussion: quint-uiq9333.slack.com