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Abstract:	This report describes the goals of SP1 for the ramp-up phase, and the methods it will use to achieve these goals. The topics covered include cooperation with other subprojects, deliverables, and methods. The report provides a timeline for the planned work, describes indicators for measuring progress and provides target values for the indicators

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1. Executive Summary

This report, entitled '*Methods, indicators of progress and target values for the mapping of mouse brain*', is the Month 6 deliverable of HBP Subproject 1 (SP1) 'Strategic Mouse Brain Data'. SP1 contributes to HBP Strategic Objective 3: Research for future versions of the platforms. The goals of the subproject are to generate strategic mouse data required for reconstruction of the mouse brain in computer models and simulations, to align mouse data sets with equivalent data sets for humans and to create the required theoretical frameworks and scientific and technological capabilities.

The subproject is divided into two general areas of experimental research: molecular biology, led by the University of Edinburgh (P71, UEDIN, Grant) and cellular biology led by the Universidad Politécnica de Madrid (P59, UPM, DeFelipe). SP1 is directly linked to other subprojects, especially SPs 2 and 5.

Primary methodologies for mapping the mouse brain will deliver transcriptome data at the level of the single cell, proteome, synaptome and connectome. Secondary methodologies will deliver electrophysiological, behavioural, vascular and glial cell data. This report will describe these methodologies in detail, specify indicators of progress, and defining target values for the indicators. Progress in methods development will be measured in terms of *categorical and aggregate stage indicators*. Progress in data generation will use *numerical indicators* appropriate to the specific classes of data in question.

2. Scope and Purpose of This Report

The HBP goal of reconstructing the human brain in computer models and simulations requires a detailed understanding of the brain's molecular and cellular components and their interactions. However, technical and ethical considerations place severe limitations on the data it is possible to collect in humans.

To circumvent this difficulty, the HBP will build brain-building tools that leverage available experimental data to reconstruct aspects of the brain where experimental data is not available. To develop, test and refine this approach, the HBP will use data describing the molecular and cellular organization of the mouse brain - a choice motivated by the huge volume of data already available or likely to become available in the near future. In this setting SP1 will generate strategically essential data unlikely to come from other sources.

This report will provide a detailed description of the methods to be used and developed, specify indicators of progress and define target values for the indicators. This document comprises the following sections:

- Chapter 3: "Purpose of HBP SP1": this section describes the aims of the subproject, collaboration with others SPs, and the deliverables the subproject plans to during the Ramp-Up phase.
- Chapter 4: "Organisation of SP1": this section outlines the way the subproject is organized and its links to other SPs and HBP central management.
- Chapter 5: "Process of Mapping of the Mouse Brain in SP1": this section provides a detailed description of the methods that the subprojects will use and of related work on methods development
- Chapter 6: "Key Performance Indicators for SP1": this section defines medium and low-level indicators for measuring progress in the subproject and target values for the indicators.

3. Purpose of HBP SP1

The aim of SP1 is to acquire strategic datasets describing the molecules, cells, and cognitive capabilities of the mouse brain and align these with information on the human brain. Assembled data on the cellular and molecular organisation of the brain will set a precedent for the comparison of mouse-human systems, allowing the reconstruction of models and simulations of the brain across all its levels and functions.

Data gathered at the molecular level will reveal the transcriptome and proteome of cell types present in the brain. The main focus will be on single cell transcriptomics and synaptic proteomics - the molecular foundations underlying nervous system function. Datasets on the cellular organisation of the brain will identify the connections between nerve cells and the organisation of brain circuits. Mapping of glial cells and the vascular system will provide a comprehensive architecture for brain simulation. These data will be integrated with the HBP Mouse Brain Atlas, providing a foundation for the integration of community data and the derivation of general principles of structural organisation that are essential for the reconstruction of mouse brain models.

Data collected and generated in SP1 will enable us to decipher the molecular structure of the human brain and map the circuits of nerve cells. Combining the data and generating detailed computer models and simulations of the brain will allow neuroscientists to comprehend the connections that link genes, molecules and cells to human cognition and behaviour—and eventually, to disease.

3.1 Cooperation with Other SPs

- SP2: SP1 collaborates with SP2 in task T2.1.3: Numbers and distribution of neurons and glia in the human brain (WP2.1). In particular, SP1 will produce ultra-high resolution datasets via the implementation and validation of automated counting methods to determine numbers and distribution of neuron and glia in different human neocortical areas.
- SP5: SP1 contributes to SP5's Brainpedia (WP5.1, Task: 5.1.5: design and operation of the HBP Brainpedia) and Mouse Brain Atlas (WP5.5, task 5.5.1: The mouse Brain Atlas) by providing them with data generated at molecular and cellular level. Additionally, SP1 is actively collaborating with SP5 in task T1.2.3: Numbers and distributions of excitatory and inhibitory neurons and glia' in the development of the method '*Automated 3D segmentation with confocal microscope*'. In particular, this collaboration has been established to develop the 3D segmentation algorithm (WP5.2: Tools for Structural Data Analysis (Peña, MIDAS-UPM and Fua, CVL-EPFL), and the application of spatial statistical techniques (WP5.4: Predictive Neuroinformatics, T5.4.2: Neuronal Structural Design and Predictions, Larrañaga, CIG-UPM).
- SP12: since SP1 includes animal experimentation in WP1.1 and WP1.2, ethical issues are involved. From the beginning of the project, SP1 has established direct communication with SP12 through WP1.4. Permits, consent forms and other required information have been provided to ensure compliance with EC regulations. This communication will continue throughout the project.

- SP13: SP1 collaborates with SP13 on the issues outlined below:
    - Project Governance: SP1 is represented in the governance bodies by its Co-directors
    - Education Program
    - Competitive Call Scheme
    - Management and coordination structures: SP1 is actively collaborating with SP13 through WP1.4.

3.2 SP1 Deliverables

During the period M1-M30, SP1 will complete 4 deliverables of which this is the first. In month 12, D1.4.2 will provide a report including a detailed account of the implementation and validation of the methods described in D.1.4.1.In months 18 and 30, D1.4.3 and D1.4.4 will deliver two data packages of mouse data. Both packages will include data to be deposited in the HBP Mouse Brain Atlas.D1.4.4 will also include modelling data.

4. Organisation of SP1

SP1 is made up of four work packages (WP). The first three involve research and technological development activities (RTD, WP1.1-1.3). The focus of two of these will be on acquiring transcriptome and proteomic data, and data on the cellular and synaptic organisation and the brain's vascular system. The aim of the third WP is to integrate the data gathered in the first 2 WP with the HBP Mouse Brain Atlas, providing a foundation for the integration of community data and the derivation of general principles of structural organisation essential for the reconstruction of mouse brain models. The aim of the fourth WP is a scientific coordination WP (WP1.4), which monitors the progress and quality of outputs from WP1.1-1.3. All SP1 tasks have started in M1 and will last 30 months with an allocated budget for this period that amounts to EUR 4,952,290. In SP1, There are 8 beneficiaries involved in five Member States of the EU and one in China. SP1 organisation and connections with other divisions and SPs within the HBP are displayed in Figure 1:

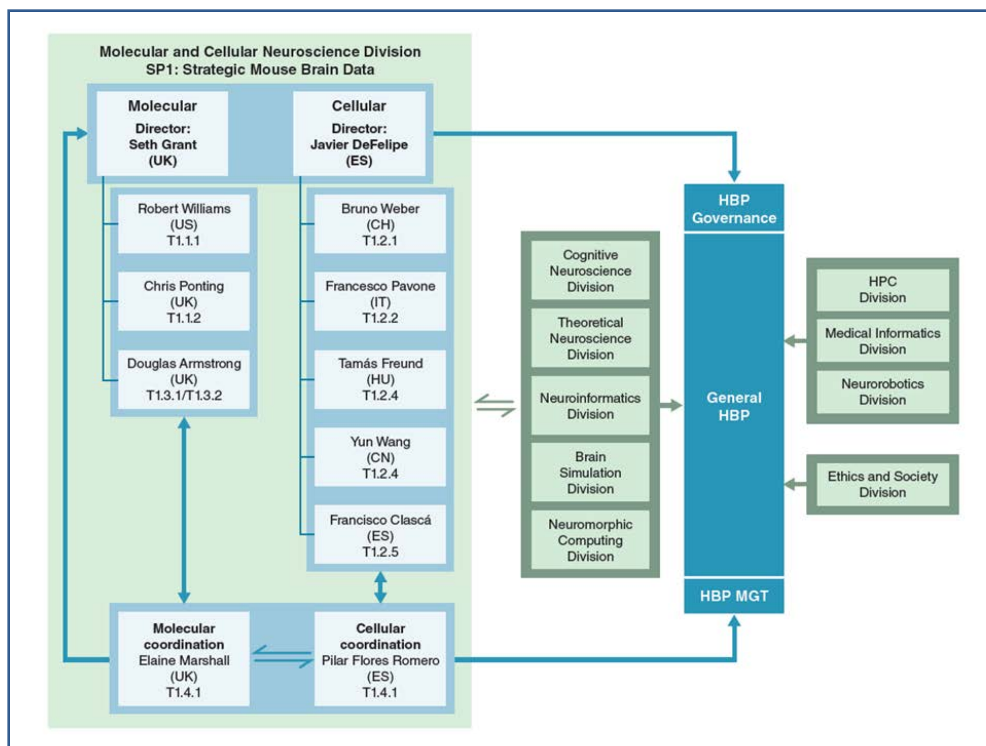

Figure 1: General scheme of SP1

5. Process of Mapping the Mouse Brain in SP1

5.1 Description of Key Methodologies

The technologies necessary to acquire single cell-type data, such as gene and protein expression maps, macrostructure, vasculature, cells and synapses of the mouse brain are in development under the SP1 framework. Scientists within SP1 are using state of the art techniques at the cellular and molecular levels to address these fundamental challenges. We summarise our primary methodologies below.

5.1.1 Transcriptome

5.1.1.1 Quantifying transcriptomes of different cells (T1.1.2)

Single cell isolation: development of a protocol for preparing and performing RNA-sequencing from single cells. Several methods are being simultaneously investigated for possible use in acquiring single neuron transcriptomes from compartments of at least three mouse brain structures: neocortex (layers I-VIb), hippocampus (CA1, CA2, CA3 and dentate gyrus), and cerebellum (Purkinje and granular layers). Our first method relies on sorting individual dissociated cell bodies or nuclei using fluorescent tags whereas others use fresh wild type tissues without special molecular tags. The first approach involves seventeen validated transgenic mouse lines in which individual neuronal types are tagged by the expression of a fluorescent protein. Our second approach expands the number of neuronal types that can be sorted, and importantly allows for translation into human tissue, by using 40 antibodies carefully chosen to selectively target neurons in each of the above compartments. Sixteen of these are additionally marketed as working with Fluorescence-Activated Cell Sorting (FACS). A third "shotgun" unlabelled approach to single cell RNA-Seq expands the number of cell types that can be sorted (essentially any cell comprising >0.1% of the population), and allows for translation into human tissue as well. A final approach addresses challenges in sorting neurons from adult tissues by instead sorting fractionated nuclei. SP1 scientists continue to compare the relative merits of different cell sorting platforms and methods of barcoding and parallelisation. As the state of the art in this field is rapidly advancing, the particular combination of experimental methods employed, the depth of sequencing, and the numbers of cells of each type sequenced will be chosen to optimise the usefulness of the overall dataset to HBP modellers and the neuroscience community.

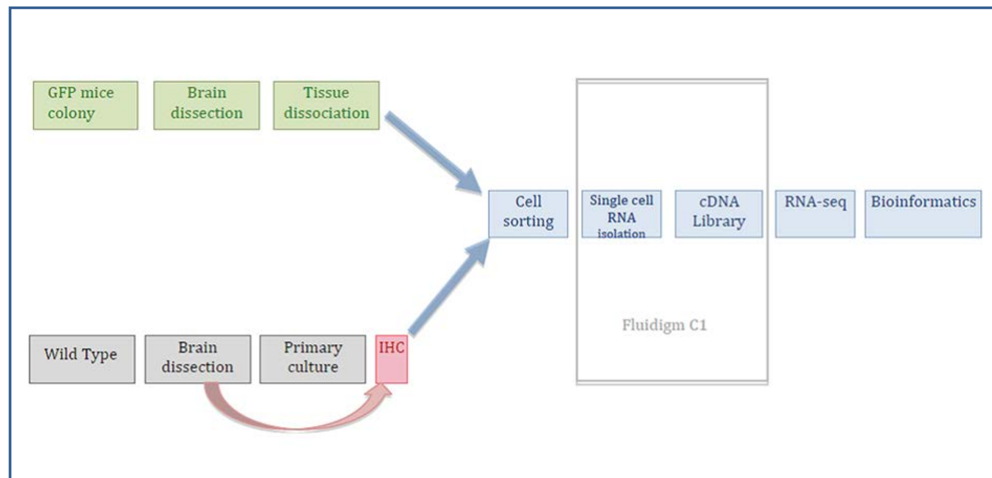

Figure 2: Single-cell RNA-sequencing initial workflow

Single cell transcriptomics analysis. Pilot transcriptome datasets have been generated from cell types as proof of principle and analysis is underway. As proof of principle, pilot transcriptome datasets have been generated from individual iPSC-derived neurons with two or three copies of chromosome 21, but which are otherwise isogenic. Although elevated expression from chromosome 21 in the trisomy can be seen among these cells, the variability in these data suggests that increased numbers of cells will be required to obtain robust information at the single gene level. Furthermore, considerable variation among samples in 3' sequencing bias suggests that 3' tag sequencing with unique molecular barcodes will be preferable to full length RNA-seq for assessing expression level. As has been done here, analyses of small pilot datasets will continue to guide experimental design before the sequencing is scaled up.

5.1.2 Proteome

5.1.2.1 The cellular and synaptic proteome (T1.1.3)

Optimisation of synaptosome and PSD complex isolation. The Grant Lab has several well-established protocols for the isolation of synaptosomes and PSD proteins from mouse and human brain tissue. We have invested considerable effort in optimising efficient protocols for high throughput isolation of PSD protein fraction from mouse and human brain tissue of improved quality and purity. Once finalised, these optimised methods will allow 16-32 'clean' pure PSD protein preparations per day, and for 8 samples to be prepared for direct analysis of PSD protein samples by mass spectrometry per day. An LC-MS/MS-compatible method that is free of detergents, salts, lipids and which yields a minimum of 20ug of protein is required for MS-based proteomic profiling. To perform various biochemical analyses on 'functional' synaptosomes and neurosomes, new extraction methods and more rapid Percoll gradient protocols are under development. These methods will provide the ability to perform High-Content Screening (HCS) and drug screening assays on synapses and neurosomes isolated from mouse brains expressing fluorescently tagged PSD proteins.

Western Blotting, imaging and quantitation. The Grant Lab has established a high-throughput digital acquisition western blotting system to allow for both visualisation and quantification of proteins and protein complexes by simple SDS-PAGE, native protein complex screening and/or 2-Dimensional protein complex profiling. Using multiple Novex XCell Surelock gel running tanks simultaneously we aim to achieve the capability of running

32 SDS-PAGE gels, or 16 BN-PAGE gels per day. We have incorporated the semi-dry transfer system that has also greatly increased our proteomic throughput allowing for 32 transfers per day. We are optimising our protocols for the Odyssey Fc imager which is capable of acquiring data from near infrared fluorophores bound to target proteins yielding more accurate quantitation of protein levels due to the linear and constant signal from fluorescent molecules compared to substrate-dependent classic western approaches, i.e., chemiluminescence using horseradish peroxidase (HRP).

Mass spectrometry analysis of PSD protein samples. Once a robust and reproducible sample preparation method has been developed, a series of pilot MS-based studies will be performed on mouse tissue and human brain material. The goal of these initial experiments will be to optimise the label-free quantitative proteomic analyses of PSD proteins isolated from human and mouse brain tissue using an HPLC coupled to an LTQ-Orbitrap XL (Thermo). Using this label-free MS platform, we will be able to analyse 176 PSD preparations by September 2014. A second cohort of 176 PSD preparations will be subjected to MS-based analysis by December 2014. The large datasets acquired will be subjected to rigorous bioinformatic analysis, including gene annotation and disease association/relevance.

Analysis of human brain tissue samples from the MRC Edinburgh Human Brain Bank. The Grant lab is undertaking high-throughput screening of the human brains currently available through MRC Edinburgh Human Brain Bank. Robust and reproducible screening protocols have been developed and implemented, as has an initial pilot study in which 10 human brains have been successfully screened.

5.1.3 Synaptome

5.1.3.1 Numbers and distributions of excitatory and inhibitory neurons and glia (T1.2.3)

Automated 3D segmentation with a confocal microscope. We are developing a new 3D segmentation algorithm that currently allows neurons (stained with anti-NeuN antibody) to be automatically segmented in 3D from image stacks obtained by confocal microscopy, providing accurate data concerning their spatial distribution and size. This method is now being adapted for its use in the 3D segmentation of all cells in sections stained for DAPI (which labels the nuclei of both neurons and glia). This work is mostly being performed in collaboration with SP5 (WP5.2: Tools for Structural Data Analysis (Peña, MIDAS-UPM and Fua, CVL-EPFL). The 3D segmentation algorithm generated will be used, along with spatial statistical techniques in collaboration with SP5 (WP5.4: Predictive Neuroinformatics, T5.4.2: Neuronal Structural Design and Predictions, Larrañaga, CIG-UPM), to characterise the density and principle patterns of spatial distribution of (i) different subpopulations of GABAergic inhibitory neurons and (ii) subpopulations of excitatory neurons. The cerebral cortex and other major brain regions will be characterised. For this purpose we have started to use both wild-type mouse brain tissue stained immunocytochemically for markers for selective neuronal populations, and brain tissue from transgenic mouse lines that express GFP in specific neuronal populations. These colonies have already been established at the animal facility of the Cajal Institute (CSIC, Spain).

5.1.4 Connectome

5.1.4.1 Morphological analysis of neurons and glia (T1.2.4)

3D reconstruction with Neurolucida (Hippocampus). In order to obtain morphological reconstructions from pyramidal cells and different types of inhibitory neurons in the mouse hippocampus, we carry out whole-cell patch-clamp recordings in the CA3 area of 600

micrometre thick in vitro hippocampal slices of 8-week-old Black6 mice. Following electrophysiological characterisation, neurons are filled with biocytin. To facilitate sampling of specific cell types we use, where available, transgenic animals expressing fluorescent markers in subsets of inhibitory neurons. After preserving the slices by aldehyde fixation the intracellularly filled neurons are visualised using immunofluorescent staining against biocytin. We optimised this procedure to be able to visualise the processes of the neurons throughout the whole depth of the sections without the need of re-sectioning, thus improving the accuracy of the reconstructions as well as the throughput. This protocol allows us to scan the whole section using a confocal microscope and reconstruct the dendritic and axonal arbour of the neuron in 3D using the Neurolucida program. The structure of the dendritic tree is recorded with special emphasis on the thickness of the processes, since this is the most sensitive parameter when modelling signal propagation in model neurons. The results of the reconstructions will be forwarded to the collaborators responsible for data management in the required file formats. Recording and filling neurons in 8-week-old animals requires the fine-tuning of slice preparation procedures to increase the rate of successful visualisation. When the workflow is fully optimised, we expect to be able to reconstruct approximately 10 neurons per month.

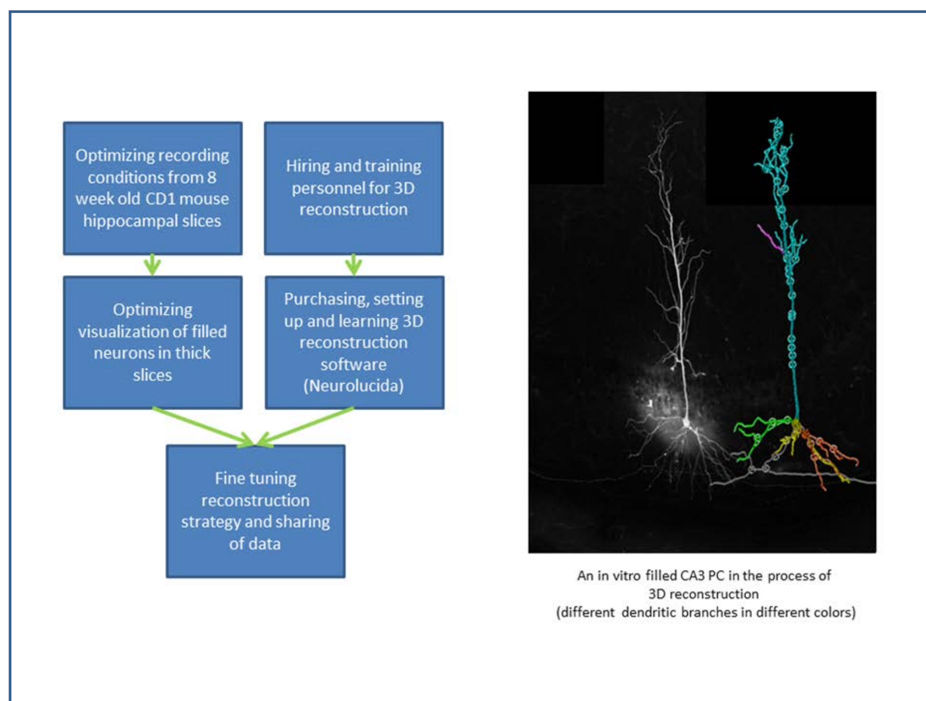

Figure 3: Scheme of the workflow for 3D reconstruction with Neurolucida in Hippocampus

3D reconstruction with Neurolucida (Neocortex): Reconstruction of different types of neurons and their synaptic connections in the mouse brain. After recording, mouse brain slices are histochemically stained, and different types of neurons and their synaptic connections are reconstructed using Neurolucida system.

Additionally, collaboration is in progress with Wuhan Institute of Chinese Academy of Sciences in China. The institute has established a new technique called MOST, by which different types of images from whole mouse brains can be scanned and integrated. The collected images include cell density, blood vessel network, and Golgi stained neurons, and single neurons specifically labelled by transgenic and virus-mediated expression of fluorescence. Hopefully, this collaboration will benefit HBP in multiple aspects. For instance, using their image data, the cell density and blood vessel networks can be reconstructed and mapped at the scale of the whole mouse brain. Using image data of fluorescently labelled neurons, the neuron reconstruction can be done at a level of the whole structure, which will be much faster compared with previous techniques based on *in vivo* labelling and subsequent serial sectioning. Using image data of Golgi stained neurons of a whole mouse brain, the reconstruction of different neuron types can be systematically carried out and compared among different brain regions.

In utero *electroporation technique.* An *in utero* electroporation (IUE) method is being developed to improve the analysis of neuronal morphology and to correlate neuronal morphology with specific molecules in the mouse cerebral cortex (see Figure 4). IUE is a method that allows rapid gene delivery in a spatially- and temporally-controlled manner in the developing central nervous system. The progenitor cells in ventricular zone carrying the DNA will undergo neurogenesis, migration, and final differentiation to become mature neurons positioned in distinct cortical layers according to their birth date. In addition, by controlling the direction of electroporation, a specific cortical area can be targeted. IUE is being used to study the development of neuronal morphology and the correlation of neuronal morphology and specific molecules in the mouse cerebral cortex. Using null mutant mice and by overexpression with this technique, we are studying the function of a layer specific transcription factor in regulating the physiological properties of cortical pyramidal cells (PCs). It was found that the target gene promotes basal dendritic growth and regulates intrinsic electrophysiological and synaptic properties of layer 5 PCs in mouse cortex.

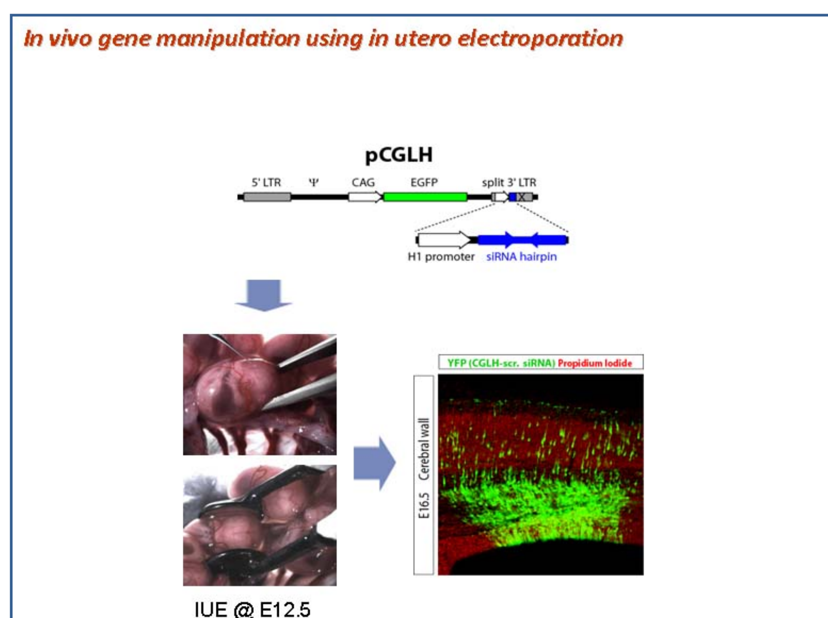

Figure 4: Scheme of the *in utero* electroporation (IUE) method

5.1.4.2 Principles of axonal projections (T1.2.5)

Axonal projections analysis. Research efforts have been mainly focused on setting up and refining the methods and instrumentation for developing this task on seven specific areas.

Three pilot experiments have been conducted to adjust the optimal brain tissue processing protocols to allow quantification at the optical/electron microscope levels (both standard and FIB/SEM) of anterogradely labelled thalamocortical axon boutons and synapses.

Two independent lines of instrumentation have been tested for achieving efficient visualisation and generation of 3D digital analysis on the complete morphologies of individual GFP-labelled axons on whole mice brains. First, the adequacy of light-sheet fluorescence microscopy (LSFM) on chemically cleared whole cerebral hemispheres is being explored in collaboration with LENS. Several mice brains injected in our lab are being examined at LENS. Second, joint experiments have been conducted and established a collaboration with TissueVision Inc. (Cambridge MA, USA) for designing and implementing technical modifications to the first commercial serial two-photon tomography scanner (STP; Tissue Cyte1000) to make it able of working in supervised mode for directed high-resolution Z scanning. STP has been applied for 3D imaging of GFP-labelled individual projection thalamocortical mice axons.

Importantly, our STP trial experiments have revealed that, even under the best epifluorescence optics and working distance conditions, the fluorescent signal coming from the thin distal segments of a single Sindbis-transfected axon are too weak for detection and imaging. Our interpretation is that weak fluorescent staining results from the relatively quick cytopathic effects of the Sindbis promoter, which kills the infected neuron in about 72 hours, thus precluding sufficient labelling saturation of distal axonal branches. In fact, we and the only other lab in the world that uses this technique (Dr. Takeshi Keneko in Kyoto University, Japan) had always relied on tissue sectioning and immunolabelling against GFP, not on direct fluorescent imaging, for visualising distal axonal processes.

Since techniques for the efficient acquisition of 3D fluorescent imaging (Laser Sheet microscopy or STP) are rapidly improving and may become available for the SP1, the development of innovative strategies for obtaining intense fluorescence in the distal axons of individually transfected neurons have been decided. Specifically we have begun testing adenoassociated viral vectors. We are currently testing AAV1.hSyn.eGFP.WPRE.bGH pseudoviruses and vector plasmids that we have obtained from the University of Pennsylvania Vector Core.

To increase the spatial precision and efficacy of our single-neuron labelling by transfection with Sindbis viral vectors, a new electro-osmotic procedure has been developed. We are currently writing the corresponding methodological report for publication in a science methods journal. In parallel, experiments to explore the feasibility of combining single-cell extracellular recodings with Sindbis RNA electroporation have been started. If successful, this technique could be crucial to correlate cell morphologies and functional profiles. Besides, a systematic population-level quantitative analysis has been started of thalamocortical afferents to primary and higher order visual areas in C57BL6 mice.

The use of stereological measurements has been tested on the axonal length and bouton number on the various terminal arborisations of a single projection axon in different brains regions. The optical fractionator and virtual planes methods are applied using an Olympus CAST-GRID Advanced Stereology System. This procedure may turn out to be an efficient

compromise for generating precise data on a large number of local arborisation morphologies of complex multi-branched projection axons.

5.1.4.3 Synapse maps of the mouse brain (T1.2.6)

Method of automated 3D counting of puncta. New methodology is under development for automated 3D counting of fluorescent puncta in stacks of confocal microscope images. This work has been performed in collaboration with Seth Grant's laboratory-UEDIN (WP1.1 - Mouse Brain Transcriptomics and Proteomics). Specifically, we have used genetically modified PSD95 EGFP mice to develop a novel 3D quantification methodology to analyse the number, distribution and size of the PSD95 protein, which is one of the main components of the postsynaptic densities. We are currently working on an automated counting process using 3D connected components. This technology will also be applied to the characterisation of the density and principles of spatial distribution of excitatory and inhibitory axon terminals in different brain regions using transgenic mouse lines with GFP labelling proteins that label glutamatergic and GABAergic terminals. Breeding animals have recently been acquired, and colonies for these transgenic animals have already been established at the animal facility of the Cajal Institute.

3D FIB-SEM Technology: Improvement of perfusion-fixation of brain samples to be used for confocal and FIB/SEM imaging. Intravascular perfusion with paraformaldehyde is performed so that it is adequate for conventional light and confocal microscopy. Sections of the same tissue to be used for electron microscopy (FIB/SEM) have been improved by postfixation with osmium tertroxide and potassium ferricyanide in a microwave oven. This technique renders a better contrast and a higher signal-to-noise ratio. Secondary methodologies that are currently being used provide data to be integrated with the transcriptome, proteome, synaptome and connectome data. These methodologies are summarised below.

5.1.5 Neuro-vascular-glia

5.1.5.1 Detailed anatomical map of brain vasculature (T1.2.1)

3D reconstruction with X-ray tomographic microscopy. A method has been developed which allows for scanning the entire mouse brain vascular system at sub-micrometre resolution in only one day. Members of our subproject met several times in Zurich and Villigen at the synchrotron beam line, which will be used to acquire synchrotron radiation, based X-ray tomographic microscopy (srXTM) images of the entire mouse brain. The focus of our initial efforts is on establishing a robust workflow from sample preparation and data acquisition to data analysis. The beamline headed by the consortium member Marco Stampanoni) has recently been further improved, particularly with respect to the speed of acquisition. Currently, we are able to image 1x1x1 millimetre at 700 nanometre resolution within one minute. As a consequence, the time required to scan the entire mouse brain vascular system at sub-micrometre resolution dropped to approximately one day.

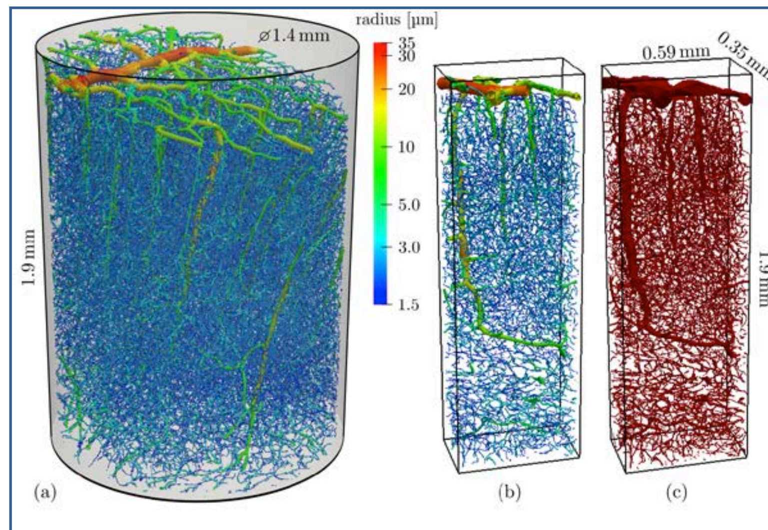

Figure 5: Vascular network of the somatosensory cortex

(a) Vectorised model reconstructed from 3-D high-resolution imaging data acquired by synchrotron radiation X-ray tomographic microscopy (srXTM) of a cylindrical sample with a volume of about 2.8 mm3. (b) Cubic subregion of (a) containing a pial arteriole at the cortex surface (top) with a penetrating arteriole orthogonally plunging into the cortex. The vasculature is visualised as vectorised model (b) and surface mesh (c). The vessel radii in (a) and (b) are color-coded on a logarithmic scale. Taken from: Schneider et al., submitted.

3D reconstruction with serial two-photon microscopy. A technique based on serial two-photon microscopy (s2pm) is currently being carried out to reconstruct large murine cortical samples successfully. As a parallel and complementary approach to srXTM, we will also work on serial two-photon microscopy (s2pm) together with David Kleinfeld, who has successfully reconstructed large murine cortical samples. A meeting was held in December 2012 in Zurich and a further meeting was held in February 2014 to set up a detailed workflow, which includes a comparison between srXTM and s2pm.

5.1.6 Cellome

5.1.6.1 Whole brain cell distributions (T1.2.2)

3D cell localisation with light sheet microscope. A novel state of the art approach for 3D cell localisation and analysis of data has been produced using new light sheet microscope with improved features: aberration correction, high-speed imaging, Bessel beam double-side illumination, multi-colour imaging. This new technology will be used to produce maps of cell distributions throughout the whole mouse brain. This new microscope will guarantee imaging of a whole mouse brain with submicron (~6-700 nm) resolution in 8-10 hours, producing a dataset in the 10 TeraBytes range per mouse brain. To store and analyse this huge amount of data, we set up a high-speed (10 Gbit/s) direct link to CINECA. Several tools to manage and inspect the data are already available (such as TeraStitcher and TeraFly) and can be used either in line modality or in remote desktop modality. Other tools for the extraction of information (like cell counting and localisation) are almost ready to be used. We are also preparing exhaustive documentation about the microscope and the data analysis in order to allow scientist within HBP to come and use the apparatus.

CLARITY. CLARITY protocol has been reproduced for mouse brain with intrinsic fluorescence (i.e. without immunohistochemistry). It seems quite stable in our hands, and we have found various alternatives to the very expensive FocusClear as imaging solution. We are in the meanwhile reproducing CLARITY immunohistochemistry on thick (~`500 microns) mouse brain slices. This will allow better integration with other data generated within the consortium. The light sheet / CLARITY approach will be used starting from the first months of the next year to generate data about cell number and localisation: Purkinje cells in the cerebellum, pyramidal neurons in the cortex, GABAergic neurons in the whole brain. Mid-term measurements will concern long-range connectivity and blood vessels.

3D reconstruction with serial two-photon microscopy. A two-photon serial sectioning tomography method is currently being setting up for subregion reconstruction with high resolution. With this method the whole brain cannot be observed at full resolution (it will take months), but subregion can be reconstructed with high contrast. We have already some preliminary results, and we are also coupling this technique with mild clearing techniques (as Scale) to reduce the need for sectioning. The idea will be to use this method to image single thalamocortical axons. The whole brain does not need to be scanned, but only the region containing the axon branches. The contrast and resolution of the two-photon microscopy will allow detecting single synaptic boutons, and the speed we plan to achieve (1-2 samples per week) will allow massive data acquisition with respect to conventional neurolucida methods. We are also adapting our existing data management tool to this technique.

5.2 Timeline for the Development of Methods

Table 1: Timeline for the development of methods

SP1: STRATEGIC MOUSE BRAIN DATA				TIMELINE				
Area	Task	Method	Phase	M6	M12	M18	M24	M30
Transcriptome	T1.1.2	Single cell isolation	Research					
			Development					
			Deployment					
	T1.1.2	Single cell transcriptomics analysis	Research					
			Development					
			Deployment					
Proteome	T1.1.3	Optimisation of Synaptosome and PSD complex isolation	Research					
			Development					
			Deployment					
		Analysis of Human Brain Tissue Samples from the MRC Edinburgh Human Brain Bank	Research					
			Development					
			Deployment					
		Western Blotting, imaging and quantitation	Research					
			Development					
			Deployment					
		Mass Spectrometry Analysis of PSD protein samples	Research					
			Development					
			Deployment					

SP1: STRATEGIC MOUSE BRAIN DATA				TIMELINE				
Area	**Task**	**Method**	**Phase**	**M6**	**M12**	**M18**	**M24**	**M30**
Synaptome	T1.2.3	Automated 3D segmentation with Confocal microscope	Research					
			Development					
			Deployment					
Connectome	T1.2.4	3D reconstruction with Neurolucida (Hippocampus)	Research					
			Development					
			Deployment					
		3D reconstruction with Neurolucida (Neocortex)	Research					
			Development					
			Deployment					
		In utero electroporation technique	Research					
			Development					
			Deployment					
	T1.2.5	Axonal projections analysis	Research					
			Development					
			Deployment					
	T1.2.6	Method of automated 3D counting of puncta	Research					
			Development					
			Deployment					
		3D FIB-SEM Technology	Research					
			Development					
			Deployment					

SP1: STRATEGIC MOUSE BRAIN DATA				TIMELINE				
Area	Task	Method	Phase	M6	M12	M18	M24	M30
Neuro-vascular-glia	T1.2.1	3D reconstruction with X-ray tomographic microscopy	Research					
			Development					
			Deployment					
		3D reconstruction with serial two-photon microscopy	Research					
			Development					
			Deployment					
Cellome	T1.2.2	3D cell localisation with light sheet microscope	Research					
			Development					
			Deployment					
		CLARITY	Research					
			Development					
			Deployment					
		3D reconstruction with serial two-photon microscopy	Research					
			Development					
			Deployment					
Data aggregation, analysis and dissemination	T1.3.1	Establishment of informatics analysis and data management pipeline for linking SP1 molecular and cellular data	Research					
			Development					
			Deployment					
	T1.3.2	Development and maintenance of informatics tools allowing integration of molecular data from multiple data sources	Research					
			Development					
			Deployment					

6. Key Performance Indicators for SP1

SP1 involves methods development and data generation in the areas of transcriptomics, proteomics, synaptomics, connectomics, cellomics, and whole brain vasculature. The progress made in the development of methods will be measured in terms of categorical and aggregate stage indicators.

6.1 Categorical Stage Indicators

- Identification of mouse lines appropriate for cell-types studies: strategically relevant mouse lines suitable for single cell-transcriptomes studies will be identified and disseminated within SP1 for use in T1.1.2. It is anticipated that by month 30, 7 lines will have been identified.
- Establishment of method for single cell transcriptomics (SCTs) from well characterised neuron-types: cells will be isolated from mice identified in T1.1.1 and RNA expression data obtained of coding and non-coding RNA for major neurons in the brain. Once the methodology has been established and validated, it is projected that: 18 SCTs from neocortical neuron types will be quantified by 30 months; 8 hippocampal neuron types with first draft SCTs will be available by 30 months; 2 cerebellum neuron types SCTs will be quantified by 30 months.
- Development and implementation of proteomic techniques allowing the identification of complete sets of synaptic protein complexes: The expected planned numbers of this activity for each 6-month step is 10, 15, 20 and 25 for M12, M18, M24 and M30, respectively
- Reconstruction of the brain vascular system at submicron resolution: 3D reconstruction of the entire macrostructure and vascular system will produce detailed maps of the mouse brain vasculature. In month 30, we will generate 6 detailed maps.
- Acquisition of brain scans for labelled cells: brain scans generated from ultra-microscopy will provide numbers, distributions and densities in different brain regions to distinguish between different types of neurons. Generation of the first cell distribution map of the whole mouse brain is expected in month 18, with up to 2 more cell distribution maps expected in month 30.
- Establishment of automated methods to generate maps of inhibitory and excitatory neurons and glia: automatic counting methods will be applied to obtain numbers and distributions of inhibitory and excitatory neurons and glia to generate synaptic maps of individual identified neurons from different areas of cortex. The number of synaptic maps scheduled is 12, 24 and 60, in months 18, 24 and 30, respectively.
- Reconstruction and classification of mouse neurons and glia: morphological analysis of neurons and glia will be carried out using reconstruction and classification techniques. The numbers of cell morphologies reconstructed in hippocampus and neocortex are expected to be around 20 in M12, increasing to up to 70 in M30.

- Tracing of axonal projections: general principles for axonal projections will be defined with data generated by tracing of axonal projections (in conjunction with the activity outlined in the bullet point below). The planned number of generated axonal projections for each 6-month step is 1, 4, 12, 20 and 30 for M6, M12, M18, M24 and M30, respectively.
- Identification and distributions of terminal axon branches and monosynaptic targets: with reference to the bullet point above the planned numbers of this activity for each 6-month step is 4, 10, 20, 30 and 40 for M6, M12, M18, M24 and M30, respectively.
- Generation of synaptic maps of brain regions (neocortex, hippocampus and cerebellum): high-resolution synaptic maps in different brain regions will be generated and validated using fluorescence microscopy and automated 3D electron microscopy (volume EM/FIB/SEM). In month 30, we will generate 2 synaptic maps.
- Establishment of informatics analysis and data management pipeline for linking SP1 molecular and cellular data: analyse and link molecular and cellular data generated in WP1.1-1.2 with Neuroinformatics (SP5).
- Development and maintenance of informatics tools allowing integration of molecular data from multiple data sources: bring together systems biology tools and sources of molecular and cellular level data such as those in EMBL programs, GeneNetworks, NIH's NIF platform, Genes-to-Cognition databases and many more.

Table 2: SP1 Categorical stage indicators for methods

Task	Activity	Target (Month)		
		6	12	18-30
T1.1.1	Identification of mouse lines appropriate for cell-types studies	Basis for methods defined Methods implemented Methods tested & validated	Methods tested & validated Strategically relevant models identified	Strategically relevant models identified
T1.1.2	Establishment of method for single cell transcriptomics (SCTs) from well characterised neuron-types	Basis for methods defined Methods implemented Methods tested & validated	Methods tested, optimised, & validated	Transcriptome data gathered from selectively target neurons
T1.1.3	Development of proteomic techniques allowing the identification of sets of brain/synaptic proteomes	Basis for methods defined Methods implemented	Methods tested, optimised, & validated	Generation of synaptic proteasome data from mouse and human

Task	Activity	Target (Month)		
		6	12	18-30
T1.2.1	Reconstruction of the brain vascular system at submicron resolution	Basis for methods defined Methods implemented Methods tested & validated	Methods tested & validated Detailed anatomical map of mouse brain vasculature	Detailed anatomical map of mouse brain vasculature
T1.2.2	Acquisition of whole brain scans for labelled cells	Basis for methods defined Methods implemented Methods tested & validated	Methods tested & validated	Generation of Cell distribution maps Cell number and localisation parameters defined in cerebellum (Purkinje cells)
T1.2.3	Establishment of automated methods to generate maps of inhibitory and excitatory neurons and glia	Basis for methods defined Methods implemented	Methods tested & validated	Methods applied in different cortical layers (I-VI) of the neocortex Generation of neocortical maps
T1.2.4	Reconstruction and classification of mouse neurons and glia	Basis for methods defined Optimisation of methods Methods tested & validated	Methods tested & validated Generation of neocortical and hippocampal cell morphologies	Generation of neocortical and hippocampal cell morphologies

Task	Activity	Target (Month)		
		6	12	18-30
T1.2.5	Tracing of axonal projections	Basis for methods defined Optimisation of methods Brain regions injected.	Single cells axonal projections traced	Single cells axonal projections traced
T1.2.5	Identification and distributions of terminal axon branches and monosynaptic targets	Basis for methods defined Optimisation of methods Brain regions injected.	Distributions of terminal axon branches and monosynaptic targets identified	Distributions of terminal axon branches and monosynaptic targets identified
T1.2.6	Generation of synaptic maps of brain regions (neocortex, hippocampus and cerebellum)	Basis for methods defined Methods implemented tested & validated	Methods tested & validated	Synaptic maps generation in the hippocampus
T1.3.1	Establishment of informatics analysis and data management pipeline for linking SP1 molecular and cellular data	Basis for methods defined	Methods implemented Methods tested & validated	Pipeline for integration of intra-SP1 data established
T1.3.2	Development and maintenance of informatics tools allowing integration of molecular data from multiple data sources	Basis for methods defined	Methods implemented Methods tested & validated	Tools available for integration of data from external sources

6.2 Aggregate Stage Indicators for Methods

Aggregate stage indicators for methods used in SP1 have been defined by identifying key elements from the activities stated previously in Table 2. The elements identified contribute to accomplishing specific goals stated in the Description of Work for SP1:

1) Acquisition of transcriptomes of major types of cells in the brain
2) Development of a first draft of the full set of protein expressed in neurons and synapses
3) Generation of a map of the brain vasculature
4) Establishment of number and distributions of cells in the mouse brain
5) Identification of morphology of key mouse neurons
6) Establishment of the principles governing how neurons project their axons within brain regions
7) Linking of all SP1 data to the HBP Mouse Brain Atlas, and assemblage of systems biology tools and molecular and cellular level data sources

Table 3: SP1 Aggregate stage indicators for methods

Goal	Activity	Indicator	Target (Month)				
			6	12	18	24	30
1	Identification of mouse lines appropriate for cell-types studies	Generation of methods	100%				
		Single-cell methods development	100%				
		Identification of models	0%	30%	45%	60%	100%
1	Establishment of method for single cell transcriptomics (SCTs) from well characterised neuron-types	Method generation	100%				
		Method optimisation	50%	100%			
		Transcriptome data generation	0%	0%	35%	70%	100%

Goal	Activity	Indicator	Target (Month)				
			6	12	18	24	30
2	Development of proteomic techniques allowing the identification of complete sets of brain/synaptic protein complexes	Generation of methods	100%				
		Methods optimisation and development	50%	100%			
		Synaptic proteasome data generation	0%	25%	50%	75%	100%
3	Reconstruction of the brain vascular system at submicron resolution	Generation of method	100%				
		Set up of the methods for reconstruction	50%	100%			
		Maps of mouse vasculature generation	0%	35%	70%	100%	100%
3	Acquisition of whole brain scans for labelled cells	Generation of methods	100%				
		Set up of the methods for 3D cell localisation and reconstruction	50%	100%			
		Generation of cell distribution maps	0%	0%	35%	70%	100%

Goal	Activity	Indicator	Target (Month)				
			6	12	18	24	30
4	Establishment of automated methods to generate maps of inhibitory and excitatory neurons and glia	Generation of methods	100%				
		Set up of the methods for 3D counting	100%				
		Maps generation	0%	0%	35%	70%	100%
5	Reconstruction and classification of mouse neurons and glia	Generation of methods	100%				
		Optimisation of methods	50%	100%			
		Generation of neocortical and hippocampal cell morphologies	10%	30%	50%	75%	100%
6	Tracing of axonal projections	Generation of methods	100%				
		Optimisation of methods	100%				
		Tracing of single cell axonal projections	20%	40%	60%	80%	100%

Goal	Activity	Indicator	Target (Month)				
			6	12	18	24	30
6	Identification and distributions of terminal axon branches and monosynaptic targets	Generation of methods	100%				
		Optimisation of methods	100%				
		Determination of the distribution of terminal axons branches and quantification of monosynaptic targets	20%	40%	60%	80%	100%
4	Generation of synaptic maps of brain regions	Generation of methods	100%				
		3D methods development	15%	60%	100%		
		Synaptic maps and ultrastructural data	0%	0%	0%	25-50%	100%
7	Establishment of informatics analysis and data management pipeline for linking SP1 molecular and cellular data	Generation of methods	100%				
		Pipeline development	0%	100%			
		Molecular and Cellular data integration	0%	30%	60%	80%	100%
7	Development and maintenance informatics tools allowing integration of molecular data from multiple data sources	Generation of methods	100%				
		Tools development	0%	100%			
		Data integration	0%	30%	60%	80%	100%

6.3 Numerical Indicators for Data Generation

We will monitor data generation using *numerical indicators* appropriate to the specific classes of data generated. Numerical indicators and targets values for M6, M12, M18, M24 and M30 are displayed in the following table:

Table 4: SP1 Numerical indicators for data generation

Task	Indicator	Target (Month)				
		6	12	18	24	30
T1.1.1	No of suitable mouse lines for cell-type studies	0	4	5	6	7
T1.1.2	No. of neocortical neuron types with first draft SCTs	0	0	6	6	6
T1.1.2	No. of hippocampal neuron types with first draft SCTs	0	0	0	4	4
T1.1.2	No. of cerebellum neuron types with first draft SCTs	0	0	0	0	2
T1.1.3	No. of brain region synapse proteomes	0	10	15	20	25
T1.2.1	No. of maps of mouse vasculature	0	4	6	6	0
T1.2.2	No. of cell distribution maps of the whole mouse brain	0	0	1	2	3
T1.2.3	No. of synaptic maps of individual identified neurons	0	0	12	24	60
T1.2.4	No. of mouse cell morphologies reconstructed (hippocampus)	0	20	50	80	110
T1.2.4	No. of mouse cell morphologies reconstructed (neocortex)	8	20-40	30-50	40-60	50-70
T1.2.5	No. of axonal projections traced	1	4	12	20	30
T1.2.5	No. of terminal axon branches and monosynaptic targets identified	4	10	20	30	40
T1.2.6	No. of synaptic maps of mouse brain	0	0	1	2	2

6.4 Risk Management Strategy

No risks have been detected during M01-M06 in SP1 tasks. If risks are detected in the following months, SP1 will follow the contingency plan stated on the 'HBP narrative' (Part B, section 1.3.2.1.7) and will report them in the next deliverable report scheduled in month 12.