Title: Mapping somatosensory connectivity in adult mice using diffusion MRI tractography and super-resolution track density imaging.

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Highlights:
Stereotypical pattern of somatosensory relay locations revealed by TDI mapping
Long-range pathways inferred by TDI were consistent with fibre-tracing methods
Structure and connectivity of TDI mapping approaching histology level

Keywords: somatosensory pathway, fibre tracking, connectivity, mouse brain, track-density imaging

Abstract (200 words)

In this study we combined ultra-high field diffusion MRI fibre tracking and super-resolution track density imaging (TDI) to map the relay locations and connectivity of the somatosensory pathway in paraformaldehyde fixed, C57Bl/6J mouse brains. Super-resolution TDI was used to achieve 20 μm isotropic resolution to inform the

3D topography of the relay locations including thalamic barreloids and brainstem barrelettes, not described previously using MRI methodology. TDI-guided mapping results for thalamo-cortical connectivity were consistent with thalamo-cortical projections labelled using virus mediated fluorescent protein expression. Trigemino-thalamic TDI connectivity maps were concordant with results obtained using anterograde dye tracing from brainstem to thalamus. Importantly, TDI mapping overcame the constraint of tissue distortion observed in mechanically sectioned tissue, enabling 3D reconstruction and long-range connectivity data. In conclusion, our results showed diffusion micro-imaging at ultra-high field MRI revealed the stereotypical pattern of somatosensory connectivity and is a valuable tool to complement histologic methods, achieving 3D spatial preservation of whole brain networks for characterization in mouse models of human disease.

1   Introduction

The rodent "whisker pathway" is an important model system, used to explore the architecture and function of local and long-range circuits in the developing, diseased and injured brain. Rodents use whisking to probe textures and surfaces of their local environment and a stereotypical link from individual facial whisker to layer IV cortical barrel comprises the somatosensory pathway, for reviews see (Fox, 2008; Petersen, 2007). The topographic map of projections, arranged in rows and arcs, is preserved in the brainstem *barrelettes* (Belford and Killackey, 1979; Erzurumlu and Killackey, 1982; Killackey and Leshin, 1975; Ma and Woolsey, 1984), the thalamic *barreloids* (Van Der Loos, 1976), and cortical *barrels* (Woolsey and Van der Loos, 1970). This study describes the first mesoscopic (20μm) mapping of trigenimo-thalamo-cortical structures and connectivity in mice achieved by diffusion MRI micro-imaging methods obtained using ultra-high field MRI.

The first sensory relay location of whisker primary afferents are the brainstem barrelettes (Ma, 1991). Second order neurons then project to the ventral posteromedial (VPM) thalamic nucleus via the lemniscal pathway (Bates and Killackey, 1985). Projections from the VPM barreloids then cluster as dense arborisations terminating in layer IV primary somatosensory cortex that appear as "barrel" shaped structures (Woolsey and Van der Loos, 1970). The structural composition and connectivity of the "whisker pathways" has been described using 2D histologic and functional activation methods. Network tracking techniques include lesion studies, (Killackey and Fleming, 1985; Killackey and Leshin, 1975); carbocyanine dyes (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine Perchlorate; DiI, DiA) (Kivrak and Erzurumlu, 2013; Seehaus et al., 2013) and myelin staining (Barrera et al., 2012); these techniques are also complemented by Lenti and Adeno-associated viral vector expression of fluorescent proteins (Aronoff et al., 2010; Dittgen et al., 2004; Wimmer et al., 2010). A range of macroscopic to microscopic functional connectivity mapping techniques include functional MRI (Kim et al., 2012; Yang et al., 1996), 2-photon imaging of electrical activity using voltage-sensitive dyes (Petersen et al., 2003a; Petersen et al., 2003b) and channel-rhodopsins to map neuronal connectivity have also been conducted (Paz et al., 2011; Petreanu et al., 2007).

Spatial preservation of whole brain networks and global architecture using 3D imaging methods delivers considerable advantage over 2D histological techniques

and partial mapping of cortical microcircuits. Diffusion MRI is an important mapping tool for tissue structure and connectivity, including information about fibre orientation and integrity and has been used to visualize tissue microstructure in humans and animal models (Behrens et al., 2003; Calamante et al., 2012a; Calamante et al., 2010; Jiang and Johnson, 2011; Kim et al., 2012; Moldrich et al., 2010).

More recently, the technique of super-resolution track density imaging (TDI) has been introduced as a means to achieve detailed visualisation of structures at spatial resolutions beyond that of the acquired voxel resolution (Calamante et al., 2011; Calamante et al., 2010). In particular, when combined with ultra-high field MRI, this technique provides very high-resolution images with rich anatomical contrast in the mouse brain (Calamante et al., 2012b).

The TDI method was recently shown to allow a clear delineation of the barrel cortex (Kurniawan et al., 2014), leading to a much improved visualisation of this discriminate structure than could be achieved with either conventional high resolution relaxation-weighted MRI or diffusion tensor imaging. In this study we sought to build on this finding, to afford 3D topographical mapping of the three major relay locations of the trigemino-thalmo-cortical pathway, and the connectivity between these locations. We report that virus-mediated fluorescent labelling of the thalamo-cortical afferents from the VPM to layer IV cortex is recapitulated using targeted diffusion MRI fibre tracking of thalamo-cortical connectivity. We present compelling evidence that the super-resolution TDI method provides a unique platform for "virtual slicing", and informs the 3D structural topography of the trigemino-thalamo-cortical pathway in mice.

2 Materials and methods

2.1 *Tissue – diffusion weighted imaging*

Adult male C57Bl/6 mice (n=3) were used for diffusion MRI tractography analysis. Animals were housed at the Florey Institute of Neuroscience and Mental Health animal facility under a 12-hour light/dark cycle with food and water ad libitum. All procedures involving mice were approved by the local Animal Ethics Committee and were conducted in accordance with the current National Health and Medical Research Council of Australia Code of Practice for the Care and Use of Animals for Scientific Purposes. All data acquired for MRI analysis were obtained using ex-vivo mouse brains. Mice were anaesthetized prior to perfusion using 0.02ml/g sodium pentobarbitone (Lethabarb). Using a Perfusion One® system (MyNeurolLab, St. Louis, MO, USA), animals were initially perfused transcardially with 0.1M phosphate buffer (PB; pH 7.4), then with either 4% formaldehyde or paraformaldehyde (for cytochrome oxidase histochemistry) in 0.1M phosphate buffer (PB; pH 7.4) containing 0.5mM Magnevist®. Brains were removed from the skull and stored in phosphate buffer containing 0.5mM Magnevist® for 4 days prior to being put into perfluoroether Fomblin Y06/06 solution medium (Solvay Solexis, Italy) for DWI.

2.2 *MRI data acquisition*

DWI data were acquired in accordance with protocols previously described (Calamante et al., 2012b; Moldrich et al., 2010). In brief, data were acquired on a 16.4 Tesla vertical bore animal system (Bruker Biospin, Germany) using a Micro2.5 gradient system and a 15 mm linear surface coil (M2M, Australia). The DWI acquisition consisted of a 3D diffusion-weighted spin-echo sequence, with TE/TR=22.8/400ms, 100μm isotropic resolution, field-of-view: $11.2 \times 19 \times 8$ mm^3 to cover the whole brain, two b = 0 s/mm^2 images and 30 uniformly distributed diffusion gradient-encoding directions (Jones et al., 1999) with b = 5000 s/mm^2 (δ/Δ=2.5/14ms), NEX=1. The images were acquired in sagittal orientation, with the rostro-caudal axis as the read direction, and the mid-lateral and the dorso-ventral as the first and second phase dimensions, respectively. Data acquisition was performed at 22°C, with a total acquisition time of ~32 hours.

Table 1 is a summary of tracking and histology methods used in this study to visualise the somatosensory structures and pathway for brainstem, thalamus and cortex, including connectivity between regions. Details regarding each of these methods and analysis are described in the following sections.

	Structure	Tractography	Histology	Age (n)
Barrelettes	Spinal Trigeminal nucleus – Interpolar part	TDI	Cytochrome Oxidase (CO)	Adult (3)
	Spinal Trigeminal nucleus - Oral part	TDI	CO	Adult (3)
	Principal sensory trigeminal nucleus, ventrolateral part	TDI	CO	Adult (3)
Barreloids	Thalamus	stTDI	CO/Nissl	Adult (3) P3 (>3)
Barrels	S1 Cortex	stTDI	Nissl Fluorescence-AAV	Adult (>3)
Pathway				
Trigemino-thalamic		Targeted tracking	DiI	Adult (3)
Thalamo-cortical		Targeted tracking	Fluorescence-AAV	Adult (3)

Table 1. Summary of the structures and connectivity visualized using super-resolution track density imaging (TDI) and histology methods to reveal architecture of the trigemino-thalamo-cortical pathway. The resolution for all TDI was 20μm isotropic and optical images were $\leq$ 1um.

2.3 AAV injection – Thalamo-cortical projections

Fluorescent labelling of thalamo-cortical afferents was done using a fluorescently

tagged adeno-associated virus (AAV) expression method. Mice aged P21 (n=3), were deeply anaesthetized using 3% isoflurane, then maintained under anaesthesia with 1-2% isoflurane and placed in a Kopf Model 900 small animal stereotaxic instrument (Kopf Instruments, California, USA) for precise skull alignment and AAV injection using a calibrated micropipette as described in Wimmer et al., 2010. AAV mOrange (100–200 nL; 1×10^{12} infectious particles/mL) (Applied Viromics, California, USA) was injected into the ventral posteromedial (VPM) thalamic nucleus using the following co-ordinates (mm): 3.0 lateral to the midline; 0.5 caudal to bregma and 1.0 depth from the pial surface. Two weeks after AAV injection, mice were perfused using 4% paraformaldehyde as described for histological processing (below). Brains were removed from skull and post-fixed for one hour, washed in PB and then placed in 20% sucrose overnight and prepared for frozen sectioning. Serial coronal 50 μm thick sections were cut using a cryostat and collected onto gelatine-coated glass slides and mounted using SlowFade® Gold (Invitrogen, USA). Fluorescent images were acquired using epi-fluorescence Nikon Eclipse E800M microscope (Nikon, Japan) (excitation wavelength 555nm) with a 4x/0.2 objective. Fluorescent images were inverted and cropped during post-processing using Adobe Photoshop CS5.

2.4 *3D fluorescent imaging of thalamo-cortical afferents using Scaleview-A2*

Scaleview-A2 method was used to facilitate deep brain imaging of thalamo-cortical projections using the fluorescently tagged adeno-associated virus (AAV) expression utilised in this study. One limiting factor for brains processed for histology is the capacity to image long-range connectivity in deep brain structures. Scaleview-A2 solution, first published in 2011(Hama et al., 2011), is a tissue clearing solution and when combined with the Olympus Scaleview-A2 immersion objective, allows deep imaging of endogenously labeled neuronal connectivity. Coronal slices from ex-vivo AAV injected brains were sliced on a vibratome at 1mm thickness and placed in Scaleview-A2 solution for 48 hours. Slices became transparent and were imaged using a Femto3D (Femtonics Ltd, Hungary) laser-scanning microscope with an InSight DeepSee femtosecond pulsing laser (Newport Corporation, California) tuned to 1050 nm and 25x/1.0 Scaleview-A2 immersion objective (Olympus, XLPN25XSVMP). A series of 3D z-stacks were collected using high-resolution and considering Nyquist criteria. Image size 516μm x 516μm x 400μm and several images were acquired that comprised projections from the external capsule to layer IV cortical barrels. Images were deconvolved using Huygens 4.4.0 (Scientific Software Imaging); alignment and movies were generated using AMIRA 5.4.3 (Visage Imaging, California, USA).

2.5 *DiI – Tracing Trigemino-thalamic projections*

The lipophilic carbocyanine dye, DiI, was used to trace afferents from brainstem barrelettes to thalamus using whole mouse brains to keep the pathway intact. In a separate group of perfusion fixed mice (n=2) aged P21, whole brains were removed from the skull and DiI crystals placed in the principal trigeminal nucleus using a dissecting microscope and stored for several weeks in 4% paraformaldehyde at 37°C to allow anterograde tracing of trigemino-thalamic projections. Brains were then incubated in 20% sucrose solution, frozen in a mold containing 6% gelatin by placing in a bath of isopentane cooled in liquid nitrogen. Brains were sectioned at 50μm thickness either in coronal or sagittal plane on a cryostat (Leica, CM300, Germany). Sections were then mounted on gelatin-coated slides and immediately imaged using

epi-fluorescence on a Nikon Eclipse E800M microscope (Nikon, Japan). Fluorescent images were inverted as described for AAV label.

2.6 *Histochemistry - Cytochrome Oxidase and Nissl staining*

Cytochrome oxidase (CO) histochemistry was used to label the mitochondrial enzyme activity in trigeminal afferent arbours and their postsynaptic target neurons (Chiaia et al., 1992). CO was done using paraformaldehyde fixed tissue from additional C57Bl/6J mice aged P35 due to reduced CO activity after MRI processing. In addition, mice aged P3 were also included in the study to obtain clearer barreloid detection using CO (minimum n=3 each age). Following perfusion, brains were removed from the skull, cryo-protected and sectioned at 25-50μm thicknesses on a cryostat in the coronal or sagittal plane. CO staining, previously described by Wong-Riley (1980), was done using a fresh solution containing 0.6 mg/mL Diaminobenzidine, 0.3 mg/mL Cytochrome C (Code #2506; Sigma) and 45 mg/mL sucrose in 0.1 M PB. Slides were incubated at 37°C in CO solution for 1 hour. Sections were washed in PB three times and then air-dried before clearing in histolene and mounted with DPX (Code # 101979; Merck Millipore) and cover-slipped. CO stained sections that contained spinal trigeminal nucleus, interpolar part, oral part and principal sensory trigeminal nucleus, ventrolateral part as described by Paxinos and Franklin (2004) were imaged using a Nikon Eclipse E800M microscope (Nikon, Japan) with a 4x/0.2 objective. Nissl staining was acquired for n=2 adult mouse brains and were used to visualize the cellular architecture of the regions containing the barrels and barreloids. Nissl staining was conducted using cresyl violet according to Paxinos and Watson (1986) ; sections containing the barrel field, primary somatosensory cortex and ventral posteromedial thalamic nucleus were imaged using a 10x/0.3 objective.

2.7 *Fibre tracking MRI*

Fibre tracking MRI was performed using the MRtrix software package
(Brain Research Institute, Melbourne, Australia, http://www.brain. org.au/software/) (Tournier et al., 2012), as well as in-house modifications of this software package. A probabilistic streamlines method (SD_PROB) was employed in combination with the constrained spherical deconvolution (CSD) technique to model multiple fibre orientations (Tournier et al., 2007). The maximum harmonic order (*lmax*) used in this study was *lmax* = 6 (Tournier et al., 2004; Tournier et al., 2008).
Details of fibre tracking analysis:
  (i)   Whole brain fibre tracking for super-resolution TDI mapping: randomly seeded throughout the brain. Two versions were considered:
     a. Standard (full-length) tracking, where the resulting whole brain fibre track datasets were used to generate super-resolution TDI maps (see below) (Calamante et al., 2010). Unless otherwise stated, whole brain TDI maps were used to guide mask creation for structures of the somatosensory pathway, including the thalamic and brainstem nuclei. Whole brain TDI maps with 50 million tracks were generated using the following parameters: 0.01 mm step-size, 0.4 mm minimum track length (minlength); FOD cutoff 0.01; initial FOD cutoff 0.1; all other options were their default values in MRtrix.

b. <u>Short-track tracking (stTDI),</u> where the track length was constrained to a maximum of 1mm to enhance visualization of certain brain structures by only considering local connectivity (Calamante et al., 2012b). In particular, stTDI was used to visualize the posterior medial barrel sub-field (PMBSF), as previously described (Kurniawan et al., 2014); e.g. see example Figure1C. Parameters used for stTDI maps with 100 million tracks included: 0.01 mm step-size, 0.3 mm minlength; 1 mm maxlength; curvature 0.015 mm; FOD cutoff 0.01; initial FOD cutoff 0.1; all other options were their default values in MRtrix.

(ii) <u>Partial-brain fibre tracking for reduced field-of-view stTDI</u>: While the stTDI approach can provide enhanced contrast, it requires the generation of a much larger number of tracks (Calamante et al., 2012b). Furthermore, the larger the number of tracks, the greater the signal-to-noise (SNR) ratio. In contrast, standard "smoothing" methods, which increase SNR by capturing the major signal characteristics but with an associated loss of fine details (Calamante et al., 2010). In order to reduce computation time while retaining a high SNR, fibre tracking was also carried out by constraining the analysis to a partial brain region, which contains the relevant region-of-interest (ROI) plus neighbouring voxels. Note that this approach is possible with stTDI because only the local neighbourhood contributes to the image intensity (cf. TDI). Partial-brain tracking parameters were empirically optimised and described for each ROI (see sections *2.8.3-4* below)

(iii) <u>Targeted fibre tracking</u>: targeted tracking was used to investigate long-range connectivity between specified regions. This involves seeding from a ROI (usually referred to as seed ROI), and often also including target and/or waypoint ROIs. Targeted tracking was done using the second order integration over fibre orientation distributions (iFOD2) algorithm due to its improved performance in tracking curved structures (Tournier et al., 2010) and, therefore, in characterising long-range connections. The parameters used for targeted tracking between sensory relay locations were empirically optimised for each case, and described for each ROI (see sections *2.8.5-6* below). For visualisation purposes, the results from targeted tracking were also used to calculate 'visitation count' maps (Note: this is equivalent to TDI maps generated from the *targeted tracking* results rather than from the whole brain tracking results).

2.8   *TDI and stTDI*

As outlined above, the results from whole brain probabilistic fibre tracking were used to produce TDI and stTDI maps. The intensity in these maps represents the number of tracks traversing each voxel. Importantly, due to the additional spatial information provided by the continuity of the tracks, the voxels of these maps can be much smaller than those of the acquired diffusion MRI data, such that the TDI method has super-resolution properties (Calamante et al., 2011). In this study, 20μm isotropic resolution maps were generated from data acquired with 100μm resolution.

Directionally encoded color TDI maps were also generated, in which the color encodes the local fibre orientation (red: left–right, green: dorsal–ventral, blue: rostral–caudal) (Calamante et al., 2012b).

2.8.1 *Regions of interest – Trigemino-thalamo-cortical pathway*

To generate TDI maps of structural connectivity of the three major relay locations of the somatosensory pathway, and connectivity between regions, masks were manually created based on the whole brain TDI maps. These included (i) the primary somatosensory cortex barrel field; (ii) thalamus, including the ventral posteromedial thalamic nucleus (VPM) and posterior thalamic group (Po); (iii) medial lemniscus; (iv) barrelettes located in the principal sensory trigeminal nucleus, ventrolateral part and the spinal trigeniminal nucleus – oral and interpolar parts as described by Paxinos and Franklin (2004).

2.8.2 *Barrel field, primary somatosensory cortex*

To view the barrel field, the stTDI map was displayed in the horizontal (axial) plane and tilted along the rostral-caudal axis by 35-55 degrees and along the medio-lateral axis by 25 degrees, as previously described (Kurniawan et al., 2014).

2.8.3 *Thalamus – Barreloids*

Whole brain TDI with 50 million tracks showed sufficient structural detail of the thalamus and surrounding anatomical landmarks for delineation of masks for the VPM and Po sub-thalamic nuclei. Masks were manually defined for the thalamus and sub-nuclei from approximately 1mm to 3mm caudal to bregma for the mouse brain in the coronal plane, with corrections in the axial and sagittal views (Paxinos and Franklin, 2004). An example of the masks used for tractography in the thalamic regions of interest is shown in Supplementary Figure S1A-B. Tracking parameters were empirically determined using a systematic approach; examples using different track lengths are provided in Supplementary Figure S2, and Supplementary Table ST1 outlines the parameters used and the CPU processing time for each trial. Based on these preliminary analyses, the chosen tracking parameters for iFOD2 were: 0.01 mm step-size, 1mm minlength and 3 mm maxlength, 0.01 FOD cutoff, 0.1 initial FOD cutoff, 0.015 mm curvature; all other options were their default values in MRtrix. Ten million tracks were generated using partial-brain fibre tracking within the seed ROI, which included the thalamus.

MRView was used to display partial-brain tracking images, which were overlaid on the whole brain TDI map and orientated in either the horizontal or oblique horizontal plane tilted anteriorly 0-15 degrees and laterally by 15-30 degrees.

2.8.4 *Trigeminal nuclei – Barrelettes*

Whole brain TDI with 50 million tracks was used to identify the brainstem regions containing the principal and spinal trigeminal nuclei, in the coronal view, from approximately 4.6 to 7.2mm caudal to bregma. Partial brain tracking of the brainstem using iFOD2 was then used to generate 100 million tracks to reduce CPU time and increase signal-to-noise ratio for barrelette identification. Tracking parameters: 0.05 mm step-size, 0.5 mm minlength, 0.01 FOD cutoff, 0.1 initial FOD cutoff; all other options were their default values.

2.8.5 *Thalamo-cortical connectivity*

Targeted fibre tracking between the thalamus (seed ROI; Supp Fig 1) and primary somatosensory cortex (target ROI; Supp Fig1) was done using 1 million tracks with the following iFOD2 parameters: 0.02 mm step-size; 5 mm minlength; 15 mm maxlength; all other options were default in MRtrix.

2.8.6 *Trigemino-thalamic connectivity*

Sensory information from the whiskers to cortex is projected to the contra-lateral thalamus via the medial lemniscal pathway after crossing the midline. Preliminary tracking results showed few tracks reached the thalamus when generated from the contra-lateral brainstem nuclei. Therefore, due to difficulties in tracking through the decussations at the midline, we decided to delineate the ipsilateral portion of the tract only, between the medial lemniscus and thalamus. The medial lemniscus and ipsilateral thalamus were selected as waypoint and target ROI respectively, to view the connectivity between regions. The medial lemniscus pathway was delineated using the 50 million whole brain TDI map at a level of the principal sensory trigeminal nucleus, approximately 5mm caudal to bregma, and the thalamus as described above. Trigemino-thalamic fibre tracking with 20,000 tracks included the following iFOD2 parameters: 0.05 mm step-size; 4 mm minlength; 5 mm maxlength; 0.01 FOD cutoff, 0.02 initial FOD cutoff; all other options were their default values in MRtrix.

2.9 *Image processing*

Image processing was done using an Intel Xeon processor (8 cores/12GB RAM). 3D image reconstruction of the whole brain TDI maps, thalamo-cortical connectivity and movies were generated using AMIRA 5.4.3 (Visage Imaging, California, USA).

3 Results

3.1 *Barrel cortex topography identified using stTDI*

High-resolution diffusion MRI data was obtained with a signal-to-noise ratio of the b=0 s/mm^2 ~70, measured using the dual acquisition, subtraction method described by (Firbank et al., 1999). The posterior medial barrel sub-field was clearly identified in layer IV somatosensory cortex using stTDI; these results were consistent with previous findings (Kurniawan et al., 2014) (Fig 1C & D).

3.2 *Thalamo-cortical tracking using TDI recapitulates projections labelled using virus mediated fluorescent protein expression.*

We then examined the projection pattern of long-range connectivity for the thalamo-cortical projections, using targeted tracking (Fig 1B) and compared this to thalamo-cortical projection pattern visualized using AAV fluorescent expression system which shows the stereotypical topography in the primary somatosensory barrel cortex (Fig 1A). The AAV fluorescent labelling provided clear identification of axonal projections from the ventral posteromedial thalamus to the barrel cortex due to the method's anterograde specificity (Wimmer et al., 2010). Fluorescently labelled Layer IV barrels and thalamo-cortical axonal projections were visualized using coronal sections (Fig 1A). The pattern of fluorescently labelled projections was consistent with the results obtained for the 'visitation count' maps from fibre-tracking MRI (Fig 1B); tracks emerged from the thalamus and clustered together in regions corresponding to the internal and external capsules. Tracks then followed the external capsule, turning perpendicular to the sub-cortical white matter and projecting to the barrel cortex, they then separated into discrete groups or clusters within the cortex, before terminating in different cortical layers. The majority of tracks were ==within the inferred barrel septum area==, which appeared as continuous streamlines that terminated just prior to exiting or as they exited the brain. Tracking results for n=3 animals are shown in Supplementary Figure S3. An example of the 3D projection pattern (Supplementary Video V1) illustrates the 'visitation count' map at 20 μm from *targeted tracking that is* overlaid on a whole brain TDI map (slice view shown Fig 1D), and reveals the 3D pattern of the connectivity obtained after seeding the thalamus and targeting the barrel cortex.

3.3 *3D imaging of fluorescently labelled thalamo-cortical projections reveal mesoscopic fibre orientation*

We also examined the 3D projection pattern of the fluorescently marked thalamo-cortical afferents using an optical clearing method that facilitated deep brain imaging. Results for the projection pattern from the sub-cortical white matter tract to Layer IV barrel revealed the coherent fibre orientation within the barrel column (Fig 2A). Fibres are shown terminating in Layer IV barrels and contain a dense collection of randomly orientated fibres. A fluorescent image is compared to the same region for the 'visitation count' maps from fibre-tracking MRI at 20μm isotropic resolution in another brain (Fig 2B). The results show this dense region of randomly orientated fibres in Layer IV barrel core (Fig 2A) corresponded to a region containing lowest signal intensity of the barrel cortex inferred in the'visitation count' maps (Fig 2B). An example of the cell density in Layer IV barrels is also provided using Nissl staining (Supplementary Figure S4A). In contrast to the randomly orientated fibres seen in the barrel, the sub-cortical white matter tract and fibres projecting to the barrel showed coherent fibre orientation, and this coherency appeared to be reflected in the degree of signal intensity shown in the 'visitation count' maps. A 3D reconstruction of two barrel columns is shown in Supplementary Video V2.

3.4 *Visualization of the thalamic nuclei improved by increasing track length and track number*

Partial-brain tracking of the thalamus, including the VPM and surrounding area, showed increased signal-to-noise ratio with increasing track length using 1 million tracks (Supplementary Figure S2). Barreloid structural connectivity was empirically determined and the VPM barreloids were observed using 10 million tracks with track length 1-3mm (Fig 3A,B and Supplementary Table ST1). TDI mapping results for the thalamus were compared with barreloid topography identified using cytochrome oxidase stained sections, which labels the mitochondrial enzyme activity in trigeminal afferent arbours and their postsynaptic target neurons (Chiaia et al., 1992), in an equivalent brain region for young mice (age P3; Fig 3C,D). However, our results suggest that the barreloids were difficult to detect using histology in the adult thalamus, consistent with previous reports (Fig 3E,F); this is believed to be due to increased inter-barreloid connections in mature rodents (Zantua et al., 1996). Our results suggest that in contrast to histology for adult animals, our TDI mapping results from adult mice could still be used to detect the inferred barreloid rows and arcs that were consistent with the barreloid microstructural pattern revealed using cytochrome oxidase histochemistry in young (P3) mice. The high cell density of the VPM region containing the barreloids in the adult animal is shown using a Nissl stained section provided in Supplementary Fig S4B.

3.5 *Trigemino-thalamic connectivity*

Results of targeted tracking, from the medial lemniscus pathway to the thalamus (VPM), showed tracks formed a series of curvilinear rods that overlapped with partial tracking of the VPM (refer 3.4) and were consistent with the structural connectivity pattern for the VPM (Fig 4). Tracks generated after seeding the principal trigeminal nucleus and targeting the contra-lateral thalamic nucleus, were consistent with the trigemino-thalamic projection pattern also shown using the DiI anterograde labeling of projections from whole brain tracing data (Fig 5). Example of the results obtained using whole brain DiI tracing (insets Fig 5), shown as inverted fluorescent images, correspond to equivalent brain regions for the results shown for the 'visitation count' maps from fibre-tracking MRI (20 μm isotropic resolution; tracks shown in hot colors) after seeding the medial lemniscal pathway and targeting the ipsilateral thalamus (Fig 5 TDI).

3.6 *Brainstem barrelettes*

Structural connectivity of the brainstem region was obtained using 10 million and 50 million tracks (Supplementary Fig S5). However, as described for the thalamus, increasing track number to 100 million increased signal-to-noise ratio improving the inferred pattern of barrelette connectivity. An example of TDI map for 100 million tracks (Fig 6) is compared to cytochrome oxidase staining of the region for a separate group of animals. This histology method was not used to provide a direct structural correlate; rather, cytochrome oxidase staining informed the topographic patterning in the region for assessment of the inferred structure and connectivity results. TDI results for barrelette connectivity (Fig 6A, D, G) show regions of higher signal intensity positioned between the barrelette nuclei, an area that is negative for cytochrome oxidase staining (Fig 6B, E, H). Directionally encoded color TDI (DEC-TDI) (Fig 6C, F, I) illustrate the majority of tracks are in a rostral-caudal direction (blue), consistent with the trigeminal - thalamic projection pattern of the region.

4   Discussion

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This study presents data supporting the complementary role of the super-resolution TDI approach compared to histology methods for mapping brain structures and connectivity of the somatosensory pathway in mice. Our principal finding is the pattern of somatosensory connectivity revealed by diffusion MRI is consistent with the stereotypical pattern of thalamo-cortical projections, visualized using a virus encoded expression system and, in a separate set of experiments, DiI tracing of the trigemino-thalamic projections. We have mapped the topography of the three major relay locations, resulting in an unprecedented level of detail achieved using TDI mapping. Particularly, TDI provides a platform for "virtual slicing" of the brain, and while this is a recognised advantage of MRI modalities compared to histologic sectioning, we found this feature especially important in regions of complex anatomy where sub-thalamic nuclei could be seen by slicing at angles that are very difficult to achieve when irrevocably sectioning tissue for histology.

4.1 *Thalamo-cortical connectivity*

The results for targeted fibre tracking, used to identify thalamo-cortical projections, showed that after seeding the thalamus, tracks entered the cortex and coursed perpendicular to the sub-cortical white matter and clustered in areas corresponding to both the barrel and septal regions. We could not separate tracks seeded from either the VPM or Po thalamic nuclei, possibly due to convergence of thalamo-cortical projections as they travel to the cortex via larger nerve fibre bundles, for example the internal capsule (Agmon et al., 1993).

We found a higher signal intensity in the septal area of layer IV barrel cortex, compared to the barrel hollows, using whole brain tracking (current study and (Kurniawan et al., 2014)) and thalamo-cortical targeted tracking. These findings would not result from connectivity between adjacent layer IV barrels (Feldmeyer, 2012) and, although there is evidence to suggest a higher concentration of myelin within the septal area compared to barrel hollows (Barrera et al., 2012), myelin is not essential for anisotropic diffusion in nerve fibres, with evidence of minor amounts of anisotropy in rodent and piglet cortex (Hoehn-Berlage et al., 1999; Mori et al., 2001; Thornton et al., 1997). In fact, anisotropy from un-myelinated nerves in garfish (Beaulieu and Allen, 1994), rodent (Seo et al., 1999), zebrafish (Ullmann et al., 2013) and human fetal brain (Tucciarone et al., 2009) suggest the radial alignment of microstructure including cell membranes is a sufficient barrier to diffusion to result in anisotropic diffusivity. In addition, complex biological architecture may influence the degree of anisotropy, including axon thickness, density and extracellular space (Beaulieu, 2002).

We also examined the tractography results with respect to the cellular architecture of the barrel cortex. Cell density in cortical barrels is higher in the barrel wall identified in mice using Nissl staining, with a low-density barrel core that contains randomly oriented thalamo-cortical afferents (current study and (Woolsey and Van der Loos, 1970)). This anatomical arrangement was consistent with our tractography results, which showed lowest signal intensity in barrels inferred in TDI mapping. Our results for 3D imaging of fluorescently marked fibres also showed coherent fibre orientation in the sub-cortical white matter and in the projections to the barrel but at a reduced fibre density compared to the larger fibre bundles. The septum of the barrel cortex is reported to contain axons from the posterior thalamic group, with the majority of

afferents projecting past the barrels toward the pia and terminate in the septum, layers I and 5A (Alloway, 2008; Oberlaender et al., 2012; Pierret et al., 2000). Overall, thalamo-cortico-thalamic connectivity within the somatosensory cortex is complex; however, such coherent fibre orientation may provide an explanation for our tractography results. It is important to note that as high magnetic-field strength diffusion imaging methods approach histologic levels, the cellular components of brain structure and connectivity must be considered.

4.2 *Thalamic substructures – VPM Barreloids*

The thalamic sub-structures were observed by selecting an area that included the region of interest (for example the VPM) and voxels of the neighbouring region. A systematic analysis was applied to achieve improved delineation of the thalamic sub-nuclei. We found that increasing track number was a determining feature that improved visualization of the VPM region, when TDI results were compared with histology for the region. This is most likely due simply to the increased signal-to-noise ratio that is achieved in the TDI maps by the generation of greater numbers of fibre tracks from which to calculate the track density (Calamante et al., 2010). The results suggest regions with elevated signal intensity were consistent with the structural determinants of the barreloid topography when compared with Nissl and cytochrome oxidase stained sections (current study and (Land et al., 1995; Van Der Loos, 1976; Varga et al., 2002)).

The ability to observe the barreloid architecture using histology, in rodents, is age dependent and requires different cutting planes in young versus adult animals (Haidarliu and Ahissar, 2001; Kivrak and Erzurumlu, 2013; Van Der Loos, 1976; Woolsey et al., 1979; Zantua et al., 1996). In mice, Zantua, et al., (1996) suggest that inter-barreloid connectivity is highest in adults, thus making it difficult to resolve barreloids. We hypothesize that the ability to resolve barreloid structure in adult mice for the first time using TDI mapping was possible due to a level of inter-barreloid connections providing sufficient information to inform barreloid structural connectivity at super-resolution. Our TDI maps for adult barreloids were consistent with that seen in early rodent brain development, and highlight the advantage of the 3D TDI method to allow "virtual" slicing, overcoming the constraints of irreversible tissue sectioning used in histology. Overall, the MRI diffusion micro-imaging was necessary to resolve thalamic structures in mice (20μm spatial resolution for mice) compared to 200μm spatial resolution for humans (Calamante et al., 2012a); future studies are warranted to create detailed parcellation of thalamic sub-structures in mice based on connectivity pattern (Behrens et al., 2003; Calamante et al., 2012a).

4.3 *Barrelettes*

The anatomy of the principal trigeminal nucleus, as described by Ma (1991), has a two-kernel peanut shell-shape when viewed in the coronal plane. Anteriorly the waist of the shell forms the hilus, which contains the trigeminus motor nucleus. Our TDI results outline the structural connectivity of the trigeminal nuclei as well as surrounding regions, which were evident using 100 million tracks. Additional 3D features of barrelette structures were observed including the rostro-caudal extent of tracks forming cylinder-shaped tubes (data not shown)(Belford and Killackey, 1979; Ma, 1991). We used cytochrome oxidase staining to detect enzyme activity in

trigeminal afferent arbours and their postsynaptic target neurons, revealing the barrelette nuclei (Chiaia et al., 1992). In TDI, the barrelette connectivity map was seen in the coronal view in the ventro-lateral and interpolar regions as shown in cytochrome oxidase staining to demarcate the barrelettes in these regions (Li et al., 1994). Conversely, our results showed that the stereotypical barrelette connectivity map was not clearly detected using TDI in the oral part, consistent with current and previous histologic findings (Belford and Killackey, 1979; Bosman et al., 2011; Ma, 1991; Ma and Woolsey, 1984).

4.4   *Future directions*

Identification of the three relay locations (barrels, barreloids and barrelettes) in whole brain TDI permits comparison of the stereotypical pattern at all levels, each providing a common reference across multiple experiments in animal models. For example, in NMDA receptor 1- knockout mice, while the axonal projection pattern from the whiskers is reportedly unaffected, the trigeminal barrelettes fail to develop (Li et al., 1994). We expect that such a dramatic loss of structural connectivity would be visible using the qualitative methods described in this study. However, in order to make quantitative comparisons of discrete changes, more suitable methods might be necessary, given the known limitations of TDI as a quantitative tool (Calamante et al, 2010, Willats et al 2014). For example, the recently proposed Apparent Fibre Density (Raffelt et al., 2012) has been shown to be suitable for identifying abnormalties quantitatively in the presence of complex fibre architecture. Alternatively, other track-weighted imaging methods (e.g. Track Weighted Fractional Anisotropy and Track Weighted Average Diffusivity (Calamante et al., 2012b) or Average Path Length Mapping (Pannek et al., 2011)) have been shown to have better quantitative performance than TDI (Willats et al., 2014). In addition, a post-processing method designed to make "track counts" a meaningful quantitative parameter by removing sources of tracking bias (Smith et al., 2013) could be used to render TDI itself more quantitative. A recent in vivo analysis of thalamo-cortical projections in the reeler mouse suggest statistically significant changes in connectivity could be detected using high field strength (9.4T) imaging combined with diffusion tractography (Harsan et al., 2013).

Manganese enhanced-MRI (MEMRI) has also been used to map discrete thalamo-cortical projections achieving layer specific contrast (Silva et al., 2008; Tucciarone et al., 2009) and functional analysis of disease models (Daoust et al., 2014). The advantage of fibre tracing using MEMRI over ex vivo methods is the ability to map neuronal activity, which can be coupled with functional MRI data providing a valuable in vivo technique (Chuang et al., 2009). However, in order to resolve discrete circuitry and other pathways not detected using MEMRI, TDI offers a complementary approach and the capability to examine whole brain connections; as well as the possibility to achieve higher spatial resolution due to its super-resolution properties.

Previous studies of the rodent somatosensory pathway have provided greater understanding of brain connectivity; however collecting micro-circuit information is extremely time consuming (Egger et al., 2012; Meyer et al., 2010; Oberlaender et al., 2012; Tsai et al., 2009; Wimmer et al., 2010). Here, we highlight the use of TDI to

expedite whole brain connectivity data informing both structure and connectivity in mice at the mesoscopic (20µm) level. Our future aim is to use diffusion micro-imaging to identify subtle changes in network architecture in mouse models of human disease.

Super-resolution TDI has already contributed to clinical diagnosis (Barajas et al., 2013) and creation of a human brain atlas using a 7T system (Cho, 2013). Increasing use of high-field strength MRI systems in clinical and research settings coupled with imaging tools with the sensitivity to resolve microstructural connections create an urgent need to identify potential "connectome biomarkers" in animal models that can be directly translated into the clinical setting for advancement of therapeutic strategies.

5   Conclusion

In summary we have shown in regions of complex anatomy, MRI diffusion micro-imaging informed at an unprecedented level, both structure and connectivity patterns at a high concordance with histologic methods. In addition, targeted tracking TDI results presented in this study suggest a key role to reveal long-range connectivity by providing spatial preservation of whole brain networks at a mesoscopic level.

Acknowledgments

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Figure Legends.

Figure 1. *Thalamo-cortical connectivity visualized using targeted fibre tracking compared to virus mediated fluorescent protein expression.* (A) Inverted fluorescent image of a coronal section taken from brain injected at the VPM with virally encoded fluorescent tag (injection needle track marked with a double-headed arrow). The "barrels" indicated by the arrows are located in layer IV cortex and are delineated by the axon terminations of thalamo-cortical projection neurons. The dotted black line, shown in Figure 1A, is to indicate the orientation plane that allows visualization of the barrels in layer IV cortex as shown in Figure 1C & D (B) Coronal slice view of a 'visitation count' map (20µm isotropic resolution) shows extended tracking after seeding the ventral posteromedial thalamic nucleus (VPM) and targeting the cortex. One million streamlines were generated which formed clusters of streamlines in the cortex. The 'visitation count' map created in this manner is overlaid (in hot colors) on whole brain TDI for anatomical reference. The "barrel" regions (white arrows), have low signal intensity in the TDI map. (C) A tangential slice view (orientated as described in methods 2.8.2) of the posterior medial barrel subfield (PMBSF) pictured using the whole brain stTDI. (D) Image in (C) overlaid with 'visitation count' map from Figure 1B (shown in hot colors). In this plane the streamlines are localized to the barrel septum. EC; external capsule; Hp; Hippocampus; p, posterior; a, anterior; d, dorsal; v, ventral. Scale Bars 200µm

Figure 2. *Thalamo-cortical projections guided by targeted tracking and track density imaging infers mesoscopic barrel connectivity.* The inferred structural connectivity of the barrel, is consistent with the architecture of the barrel column identified by histology; in which random fibre orientation are inferred as low density regions in the TDI map (barrel) and the coherent fibre orientation is consistent with the increased TDI density in the cortex (WMT and septum). (A) High-resolution 2-photon grayscale image of thalamo-cortical projections from another animal labeled using the AAV-fluorescent expression system. This example shows the barrel column, defined by the fluorescently labeled barrel and its afferents, which has been rotated 45 degrees clock-wise to the right (cortical surface is at the top and white matter tract (WMT) at the bottom of the image). The coherent orientation of thalamo-cortical fibres is shown, which terminate as dense random arborizations forming the characteristic "barrel" structure (area indicated between *arrow heads* in both A and B). The barrel septum (arrows in A) has no fluorescent labeling. The 3D projection of the micro-circuitry of fluorescently marked thalamo-cortical afferents in the cortex is shown in the Supplementary Video V2. **(Inset in A)** Crosshairs mark the center of the barrel located in Row B; Arc 1 in a tangential slice view of the barrel field in the fluorescent image (note only 2 barrels shown). **(Inset in B)** Crosshairs indicate the barrel in an equivalent region using whole brain stTDI map that show the barrel location with respect to the whole barrel field. (B) The region identified in the inset images, is shown in the coronal slice view for results of a 'visitation count' map using (20µm isotropic resolution), after seeding the thalamus and targeting the cortex. A higher signal intensity in the barrel septum (arrows) is shown, compared to low signal intensity barrel (*region between arrow heads*).

Figure 3. *Barreloids in the thalamus inferred using TDI mapping and cytochrome oxidase staining.* The location of the barreloids is indicated in the boxed area shown in the context of the whole brain TDI maps that have been orientated to view the sub-thalamic region of interest. The slice view is orientated in either the horizontal plane, to view barreloid rows (A); or oblique sagittal orientation to view arcs (B; as described in Methods). (C,D) Example TDI map (20μm isotropic resolution) from thalamic region in an adult mouse, arrows indicate rows (C) and arcs (D) of the inferred barreloids in the same cutting plane as the examples shown for cytochrome oxidase stained sections (E, rows) and (F, arcs) using mice aged P3; the barreloid architecture is shown after labeling the mitochondrial enzyme activity in afferent arbors. (G,H) The barreloid rows (arrows) from adult mice, which are not readily seen in sections stained using cytochrome oxidase, likely due to reduced inter-barreloid connectivity in younger mice compared to adult animals.

Figure 4. *Trigemino-thalamic pathway shown using targeted tracking from barrelettes to barreloids via the medial lemniscus.* (A) The tractography results are shown after seeding the principal trigeminal nucleus (Pr5) in the brainstem and targeting the contra-lateral thalamus (Thal), tracks travel via the medial lemniscus (ML). Targeted tracks have been overlaid on whole brain TDI map for anatomical reference (viewed in the horizontal plane). (B) Targeted tracking after seeding the ML and targeting the ipsilateral thalamus, results from the 'visitation count' map is shown with directionally encoded color, white arrow indicates track direction. 'Visitation count' map has been overlaid on a whole brain TDI map to illustrate termination of tracks in the thalamus in the ventral posteromedial thalamic nucleus (VPM).

Figure 5. *Trigemino-thalamic targeted tracking and super-resolution TDI is analogous to that seen using a carbocyanine dye tracing method.* The connectivity pattern illustrated using targeted tracking and super-resolution TDI was analogous to fluorescent lipophilic dye tracing, DiI, of the medial lemniscal pathway. (A) TDI mapping of trigemino-thalamic targeted tracking shown in sagittal slice view; results of a 'visitation count' map (shown in hot colors) are shown after seeding the medial lemniscus and targeting the thalamus. Results have been overlaid on a whole brain TDI for anatomical reference (both at 20μm resolution). Photomicrographs shown on the left of the TDI map are an example of inverted images of the tracing using DiI, from a representative example of a fixed whole brain, in which trigemino-thalamic projections were labeled by placing DiI crystals in the principal trigeminal nucleus and anterograde, passive tracing of projections took place over several weeks. Inverted fluorescent images are from 50μm sections in the sagittal plane (labeled projections appear black) for the DiI tracing along the medial lemniscal pathway. DiI images correspond to equivalent brain regions shown (dashed rectangles) in the TDI map. Rostral to the right and caudal to the left of images; the plane of section is approximately 1mm lateral to the midline.

Figure 6. *Barrelette connectivity pattern in the brainstem shown using TDI mapping and cytochrome oxidase staining.* Images are shown in the coronal slice view through the brainstem from caudal (top row) to rostral (bottom row; midline to the right). (Inset in A-C) Whole brain TDI map viewed in the horizontal plane, to indicate position of coronal slice marked with yellow crosshairs. (A-C) Arrows

indicate the three anatomical locations compared using TDI and histology: Spinal trigeminal nucleus interpolar part (SpI); (D-F) Spinal trigeminal nucleus oral part (SpO) and (G-I) Principal trigeminal nuclei, ventrolateral part (Pr5VL). (A,D,G) TDI map (20μm isotropic resolution) with 100 million tracks show regions of higher signal intensity representative of barrelette connectivity pattern. Barrelette structural connectivity is more clearly defined in the interpolar part and principal trigeminal nuclei, ventrolateral part. (B,E,H) The tracking results are compared to micrographs of cytochrome oxidase to visualize barrelette topography (region of interest outlined by dotted line) in an equivalent region of another animal which show positive staining in postsynaptic target neurons of the trigemino-thalamic connections; regions without cytochrome oxidase staining correspond to nerve fibres. Barrelettes are shown in the SpI and Pr5VL. (C,F,I) Directionally encoded color track density images show streamlines surrounding the barrelettes are orientated in the rostral-caudal direction (blue), consistent with the trigemino-thalamic projection pattern in the region. Note that the topography of barrelette connectivity was not distinct in the oral part using tractography (D, F) or cytochrome oxidase histochemistry consistent with other histologic reports (E). m5, motor root of the trigeminal nerve; PCRt, parvicellular reticular nucleus; Pr5DM, principal sensory trigeminal nucleus, dorsomedial part; S5, sensory root of the trigeminal nerve; Sp5, spinal trigeminal tract.

Supplementary:

Video V1: 3D projection view of inferred thalamo-cortical connectivity. Targeted tractography results after seeding the thalamus and targeting the somatosensory cortex. The results of the 'visitation count' map (shown in hot colors), is overlaid on whole brain TDI map for anatomical reference (both 20μm isotropic resolution). Part of the rostral and caudal areas of the whole brain TDI have been removed in the 3D visualization to assist viewing the tracks origin from the thalamus located in the central region of the brain.

Video V2: 3D projection of thalamo-cortical afferents acquired using two-photon microscope. High-resolution images of the AAV mediated fluorescent labeling of thalamo-cortical afferents from the sub-cortical white matter terminating in Layer IV barrels. The coherent fibre orientation of afferents is shown before fibres terminate as randomly orientated axon arborizations forming the barrel structure.

Supplementary Figure S1. Seed and target regions of interest (ROIs) used for tractography. (A) Ventral posteromedial thalamic nucleus (green) and Layer IV somatosensory cortex (red) were identified using whole brain track-density images generated with 5 million streamlines, mask ROIs are shown overlaid on whole brain TDI for anatomical reference. The ROIs were also used for thalamo-cortical targeted tracking as described in Methods (2.7). (B) ROIs for the thalamus (yellow) and brainstem (red) shown overlaid on a whole brain TDI in the horizontal slice view and (C) coronal view for the brainstem. ROIs were used for trigemino-thalamic targeted fibre tracking and also the partial-brain fibre tracking to identify the thalamic barreloids and brainstem barrelettes.

Supplementary Figure S2. Sample TDI maps (20 μ m isotropic) of the thalamus generated after trial parameters were used to visualize inferred barreloid

connectivity. TDI maps are shown for 1 million tracks using SD_PROB and iFOD2 at increasing track length (outlined in Supplementary Table ST1). The schematic shown in the top right illustrates seed ROI and mask region used for partial-brain fibre tracking. The super-resolution TDI slice views are shown orientated in the oblique sagittal plane to visualize the barreloid arcs are indicated by the arrows in the first image, top row and arrows plus yellow circles in the last image, top row. The "best" parameters to observe the barreloids compared to histology as the benchmark were obtained using iFOD2 and track length 1-3 mm. The example gray scale image is shown of cytochrome oxidase positive staining (bottom right) of trigeminal afferents within the barreloid arcs.

Supplementary Table ST1. Summary of the tracking parameters used to visualize the barreloids. Outlined in top left column is a list of tracking parameters held constant including step size, cutoff, initcutoff. For the two algorithms (SD_PROB and iFOD2) only the track lengths were increased while the seed ROI remained constant and included VPM (schematic shown in Supplementary Figure S2). A qualitative assessment was made to compare the TDI maps with the histology images of the VPM. From this data the TDI map that showed closest representation of barreloid pattern compared to histology was achieved using medium track length ranging from minimum 1 to maximum 3mm (1-3), with at least 1 million tracks. Asterisks indicate example images that are shown in Supplementary Figure S2.

Supplementary Figure S3.   Replication of the targeted tracking results for the inferred thalamo-cortical projections. (Column A) Example of whole brain track density imaging (TDI) viewed in the coronal plane for three mice. Arrow and arrowhead show the projections within the internal and external capsule respectively in the TDI map for each animal. (Column B) The results for 'visitation count' map (shown in hot colors), when seeding the thalamus and targeting the somatosensory cortex. (Column C) Results for 'visitation count' map for inferred thalamo-cortical connectivity overlaid on the whole brain TDI map as an anatomical reference. Scale 200 μm.

Supplementary Figure S4.   Nissl staining reveals the dense cellular architecture of the cortical barrels and thalamus in the mouse somatosensory pathway. (A) Nissl stained coronal section of the primary somatosensory cortex reveals the barrels located in Layer IV (Layers I-VI are indicated by dashed lines). Barrels are identified by a high cell density in the barrel walls (arrows also shown Inset in A) with lower cell density in the central region, referred to as barrel hollow, which contain terminations of thalamo-cortical primary afferents. (B) A parasagittal Nissl stained section through the thalamus of an adult mouse shows the high cell density delineating the ventral posteromedial thalamic (VPM) nucleus containing the barreloids.

Supplementary Figure S5. Brainstem barrelette topography revealed by increasing track-density in the brainstem. (A,D,G) Whole-brain TDI map with 10 million tracks (20μm isotropic resolution) provides low signal to noise ratio of inferred brainstem structure in the spinal trigeminal nucleus Interpolar (SpI), oral (SpO) and principal nucleus (Pr5V). (B,E,H) TDI map with 50 million tracks increases signal-to-noise ratio, the barrelette topography is more clearly defined in the interpolar and principal trigeminal nuclei. (C,F,I). The TDI maps are compared to

equivalent regions of another animal using cytochrome oxidase histochemistry to detect barrelette architecture.