

Measuring macroscopic brain connections in vivo
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Brief summary: Measuring brain connections in humans continues to pose challenges despite the recent advances in MRI technology. We contrast methods used in humans with those used in animals and show the extent to which human techniques can inform us about connections despite their limitations.

Abstract

Decades of detailed anatomical tracer studies in non-human animals point to a rich and complex organisation of long-range white matter connections in the brain. State-of-the-art in vivo imaging techniques are striving to achieve a similar level of detail in humans, but multiple technical factors can limit their sensitivity and fidelity. In this review we mostly focus on magnetic resonance imaging of the brain. We highlight some of the key challenges in analyzing and interpreting in vivo connectomics data, particularly in relation to what is known from classical neuroanatomy in laboratory animals. We further illustrate that, despite the challenges, in vivo imaging methods can be very powerful and provide information on connections that is not available by any other means.

1. Introduction

Connections play a central role in brain function and, therefore, in all of neuroscience. Local intra-cortical connectivity constrains the type and nature of neuronal computations. Long-range white-matter connections allow information to be distributed across brain systems. These macroscopic connections comprise only about ten percent of the total connections in the brain [1], yet they are crucial in order to gain insight into how brain systems perform computations [2].

A wealth of neuroanatomical tools for measuring brain connections have been developed, particularly during the past fifty years. Techniques for measuring at the micro-scale (neurons, axons, synapses) are entering an industrial era, with fast automated imaging (e.g. Serial Block-faced Electron Microscopy [3]) replacing labour-intensive approaches. At the macro-scale (regions, fibre bundles), the development of neuroanatomical tracers has generated an explosion of very precise connectivity data in animal models. More recently, non-invasive in vivo imaging

methods, mostly based on Magnetic Resonance Imaging (MRI), have generated much excitement, particularly for their applicability to the human brain. Non-invasive tools have the unique ability to allow whole brain measurements of long-range connections, in addition to the potential for parallel acquisition of brain activity, behaviour, and other types of metadata that can be related to connections.

This review highlights some of the key challenges to in vivo connectomics, particularly in relation to what is known from classical neuroanatomy in animals. Compared with these ex vivo techniques, the in vivo tools are less precise and have several practical and conceptual difficulties that can limit their interpretability. However, there is growing evidence that the available tools are already providing detailed information about connections that is not available by any other means.

2. Classical neuroanatomy relevant to in vivo connectomics

The vast majority of our knowledge of white matter connections in animals comes from tracer studies. By using the natural axonal transport mechanisms to trace connections, these methods can be very precise with regard to spatial localization. Quantitation of connection strengths can be more challenging, but when achieved can be considered as providing a gold-standard in measurements of large-scale connectivity.

Decades of detailed neuroanatomical studies in animals reveal a complex organisation of brain connections, including at the macroscopic scale. Before considering the challenges faced by in vivo connectomics, it is informative to examine some "facts" that we can glean from tracer studies.

A striking observation from tracer results in macaque monkeys is the predominance of intrahemispheric cortico-cortical association connections, compared to sub-cortical and commissural pathways. This appears to be true even when restricting consideration to non-local, long-range connections. One question that remains debated is how dense the large-scale cortico-cortical network is. While some investigators report a relatively sparse network (10% according to [4]), others point to a more highly dense area-to-area connectivity matrix (66% according to [5]). If confirmed, such a high density graph implies that macaque (and presumably also human) white matter contains more than ten thousand pathways with distinct origins and terminations [6]. In sharing a restricted space, these pathways must necessarily take a variety of complex trajectories within the white matter in order to reach their targets. These can include tightly fasciculated tracts over long distances as well as dispersion and/or branching from/into fascicles. Therefore, although white matter appears to be grossly organised into a relatively moderate number of large bundles (major tracts), axons must also follow more complex trajectory patterns in order to reach their gray matter targets.

Another consequence of a highly dense network of white matter connections is that analyzing network organisation in terms of mere existence or absence of a connection (i.e., a binary analysis) becomes inherently limited. Instead, other network descriptions may be more informative, such as intra-area organisations, or quantitative information on connection "strength", such as the number/density/calibre of axons connecting two regions. Additionally, tracer studies reveal quite complex spatial or topographic organisation of white matter connections. For instance, brain

regions can display preferential connectivity to certain cortical layers [5, 7], but can also sometimes target the entire width of cortex [8]. Connections can form topographic maps, but can also form patches of varied regularity [8], with no clear relationship to cortical folds (e.g. biases towards sulci or gyri). Summarising such rich connectivity patterns in terms of an area-to-area matrix, while being a useful conceptualisation of brain networks, may hide important and functionally relevant organisation.

Examples of connection patterns that may pose challenges to in vivo imaging methods (see next section) are shown in figures 1A and 1B. In figure 1A, ipsilateral and contralateral long-range connections converge within the principal sulcus in the macaque monkey [9]. Their terminal fields can display striking interdigitation, a pattern that would need imaging at relatively high spatial resolution to be captured. In figure 1B, an example of laminar organisation of cortico-cortical connections is shown. Such pattern would require the ability from an imaging modality to separate connections to/from different cortical layers.

In terms of quantitative descriptions of connections, tracer studies point to a wide range (over five orders of magnitudes) of cortico-cortical connection strengths [10], as well as a distance rule whereby distant connections tend to be progressively weaker than short connections, following an approximate exponential law [11] (Figure 1C top). Long-distance pathways that are sparse in connection strength will presumably be particularly difficult to identify reliably using tractography. This issue is amenable to objective, systematic analysis by comparing high-resolution post-mortem tractography and quantitative tracer-based parcellated connectivity data in monkeys [12].

As mentioned before, neuroanatomical tracing is considered the gold-standard in measuring connections, as it has two important strengths. First is the low false positive rate. Detection of the injected compound away from the injection site is very strong evidence for a connection. Second is the spatial resolution, which allows the discovery of the detailed organisations shown, e.g. in figure 1. Tracer studies, however, also have several caveats, amongst which two are particularly relevant here. Assignment of the injection site to a particular brain area is not necessarily known, particularly when no clear cytoarchitectonic areal boundaries are present. This not only “blurs” the resolution in the injection site, but it also makes it difficult to combine tracer findings from different animals in order to build a normative area-to-area connectome. Another limitation is the difficulty in quantifying the connections using tracers. Retrograde tracing followed by cell counting is one option, but it can only count cell bodies, not axon terminals or synaptic strength, and therefore it is only a proxy for connection strength. Anterograde tracing can reveal the extent of terminal fields, but using these techniques to quantify connections (e.g. number of terminal buttons) is both technically difficult and vastly labour intensive.

In summary, classical neuroanatomical studies reveal that brain connections are complex, even at a macroscopic scale. Long-range connections can be nicely organised into regular bundles but can also show more complex trajectories. Terminal fields in the gray matter can have intricate organisations, such as layers, clusters, columns, or gradients. Finally, inter-areal connection “strengths”, although difficult to quantify precisely, may span multiple orders of magnitude.

3. In vivo tools: what can we measure?

Invasive tools can give us extremely precise information but mostly about animal brains. They are limited to sampling a very small percentage of brain connections even when data are combined across animals. Additionally, these tools cannot easily be used in conjunction with functional or behavioural measurements. In-vivo imaging methods, such as diffusion and functional MRI, can provide information about brain connections non-invasively. They are far less accurate but can measure millions of connections in single subjects, can be used for whole-brain longitudinal analyses, and are amenable to multimodal investigations of the human brain. In order to understand the potential of these tools, either in isolation or in combination, it is important to examine how they work and understand why and when they don't work.

3.1. Diffusion MRI & tractography: principles and failure modes

Key technological developments in MRI during the late 1980s and early 1990s engendered some powerful methods for measuring structure and function non-invasively [13, 14]. A direct consequence of these developments is diffusion MRI tractography; to date the only available tool to estimate the trajectories of brain white matter *in vivo*. Diffusion MRI is sensitive to the random thermal motion of water molecules, which is hindered by tissue microstructure. When this microstructure is organised, such as in white matter, water diffusion is anisotropic; in that diffusion is less hindered parallel than perpendicular to axons. Therefore, by measuring the orientational dependence of water diffusion, we can estimate axonal orientations *in vivo* (figure 2A). Tractography algorithms use local information on orientation to infer long-range connections (figure 2B) and allow us to perform *in vivo* "virtual dissection" of white matter bundles [15]. By repeating tractography for many brain locations we can construct a connectome, a comprehensive map of macroscopic connections as estimated by diffusion MRI.

Despite having been around for two decades, *in vivo* connectomic approaches are still in early days, considering the many technical and conceptual challenges that remain to be addressed in order to improve their interpretability. Many of the technical limitations are well known within the community of *in vivo* connectomics [16-18], and addressing them is the object of intense research (for example [19-22] for new methods or [23, 24] for new data acquisition technologies). Here we provide an overview of the conceptual challenges that tractography methods face.

A fundamental limitation of tractography is the indirect nature of the measurements. Unlike in (electron-) microscopy where individual axons can be visualised and their trajectories directly reconstructed, in diffusion MRI tractography, axonal orientation is *inferred* through the scatter pattern of water molecules. It is relatively easy to model the effect that a single axon has on the water diffusion profile (although see [25] for a more complex view). However, considering that a white matter voxel may contain hundreds of thousands of axons, unless all these axons are well aligned, the mapping from diffusion to axonal orientations is often ill-posed (Figure 2C, all these patterns are likely to give rise to the same MRI measurement). As a consequence, tractography algorithms can take "wrong turns" and produce a number of false positive and negative connections [17].

The incidence of erroneous connections in tractography is largely unknown, except when ground truth from tracer studies in the same species or brains is available [26, 27]. This question was quantitatively investigated in [27] where white matter trajectories estimated using tractography were compared to a macaque atlas [28]. It was found that the sensitivity/specificity trade off was highly dependent on the model and algorithm used in tractography, and that the optimal settings were also dependent on the white matter tract under investigation (although connections from only two cortical regions were investigated in the study). Building an accurate picture of white matter pathways requires care, and anatomically informed priors, such as where a pathway can go and where it must not, can provide important constraints for obtaining good results.

For the purpose of building a connectome, it is necessary to not only map white matter trajectories but to also map their gray matter origins and terminations. This poses a problem because often the voxel resolution is coarse relative to cortical convolutions (e.g. schematic dashed boxes in Figure 1A). Another limiting factor is a tendency for tractography-reconstructed pathways to terminate in gyral crowns, as opposed to the walls or sulci (so called gyral bias [6, 29]). This is in part due to the resolution of diffusion MRI data, and also to superficial white matter bundles (running parallel to the cortex) impeding the detection of fibres entering the cortex at sulcal fundi [29]. It has been estimated that 50% of the cortex is affected by the gyral bias, although this estimate depends heavily on the details of the tractography process. Notice that reaching the correct gray matter locus is easier for sub-cortical terminations, both because these connections tend to be organised into coherent bundles and also because the sub-cortical targets have simpler topologies compared to the cortical convolutions.

Limitations due to poor imaging resolution have the potential to be addressed (at least to an extent) in the future, but this will likely require major improvements in scanner hardware compared to what is routinely available at present. Modern acquisition techniques aim to push the boundaries of in-vivo MRI [23, 30]. Combined with more sophisticated modelling of high quality diffusion data one can markedly reduce the gyral bias [31].

Another consequence of the indirect nature of diffusion tractography is the difficulty in interpreting tractography results *quantitatively*. Diffusion MRI, is in itself a quantitative technique; it allows us to measure the apparent diffusion coefficient of water in tissue. However, the diffusion coefficient of water is far removed from what we want to infer for connectomics. Rather, we are interested in parameters that reflect physical properties of the connections (axon density, calibre, myelination). Inferring these properties from water diffusion is equally, if not more ill-posed than inferring orientation. Several groups are attempting to improve the solutions for this inverse problem using more complex modelling and/or more advanced diffusion MRI sequences [32-35].

Pending these advances in quantification of white matter connections, it is possible to calculate semi-quantitative measures that reflect interesting aspects of anatomy. For instance, an extensively used measure of white matter "integrity" is fractional anisotropy (FA) [36], which represents the (normalised) variance of the diffusion coefficient along all directions in 3D under the assumption of anisotropic Gaussian diffusion [37]. A number of experiments in animal models showed how FA relates to

myelination, membrane permeability and fibre density in white matter [38-42]. Although FA may reflect fibre integrity, it can additionally be confounded by factors that do not necessarily reflect white matter integrity such as partial volume effects or axonal orientation heterogeneity [43]. Other MRI methods that do not measure diffusion can also provide useful quantitative measurements relevant to white matter pathways. For instance, mapping of relaxation times (T1 and T2) combined with multi-compartment models can provide estimates of myelin content in the white matter [44, 45]. However, many axons in white matter are unmyelinated, and there are unmyelinated portions near the origins and terminations of myelinated axons. Lack of myelin modulates but does not eliminate diffusion anisotropy (see e.g. [38]), making this another important but complex issue to consider in quantitative tractography analyses.

Additionally, probabilistic tractography algorithms attempt to add a quantitative dimension to tractography results by calculating the probability that pathways pass through any given brain location. However, these probabilities mainly reflect our uncertainty in fitting fibre orientations to the diffusion data and building pathways through these orientation fields. Several true anatomical factors (e.g. axon density) can influence these probabilities, but other confounding factors can also contribute significantly. These include for instance the distance travelled by the pathways, their degree of curvature, and the complexity of white matter that they go through [17, 18]. Nevertheless, the relative contrast in such probability maps has been shown in many cases to carry discriminative power of borders between functionally-distinct brain areas [46].

In summary, diffusion tractography is based on an indirect mapping between the scatter pattern of water molecules averaged over millimetre-sized voxels and the micrometre-scale arrangement of axons. This makes tractography error-prone and difficult to quantify. Ongoing and future advances in image acquisition and modelling will hopefully help improve tractography both in terms of anatomical fidelity and quantitative interpretability.

3.2. Functional MRI: Inferring structure from function

Although tractography is the only available in vivo tool for inferring anatomical long-range connections, measurements of brain activity using MRI can also be used as an indirect means to assess large-scale brain connections. The essence of this type of "functional connectivity" is that brain regions that are linked via long-range connections are likely to be correlated in their brain activity patterns. Therefore, by measuring statistical dependencies in brain activity between regions, and identifying pairs of regions with strong dependencies, we can infer which regions are connected (but not the anatomical route of these connections). Typically, these types of measurements are made "at rest", without the use of an explicit task to drive brain activity. This so called resting-state functional MRI (R-FMRI) connectivity was first demonstrated in [47], where it was shown to detect strong functional connectivity within the motor system. R-FMRI has since been used extensively to study brain networks in a wide variety of experiments.

An alternative to using resting-state activity for estimating functional connectivity is to use task-induced activity, as measured by task functional MRI (T-FMRI). Given a large set of task conditions, the idea is to map the *location* of brain activity in these

conditions, and to measure spatial correlations in task response between brain regions, to produce co-activation maps [48]. The motivation for using co-activation maps as a connectivity probe is similar to that of resting-state connectivity: connected brain areas are more likely to be involved in similar tasks. A study comparing the BrainMap database (a large collection of task-based studies [49]) to R-FMRI in a small group of subjects explicitly tested the relationship between task co-activation and R-FMRI correlations [50]. Using independent component analysis, the study found remarkable similarities in several task co-activation networks and resting-state networks.

Functional connectivity has been reviewed extensively elsewhere (for example [16, 51, 52] and many more). Here, we consider the role of functional connectivity in the context of inferring information on *anatomical* connections [22], and we contrast this technique with in vivo diffusion MRI tractography.

Like tractography, functional connectivity is an indirect measurement; and is so in at least two ways. Firstly, statistical dependencies in brain activity do not necessarily reflect actual anatomical connectivity. Regions separated by intermediate connections (multiple synapses) can also appear correlated, and so can regions that receive a common driving input. A region that receives several input connections from segregated sub-networks may appear *less* connected on average due to interferences between signals from these sub-networks. This is an interesting possibility that has consequences in interpreting the discovery of "hubs" in brain networks using functional connectivity [53]. Counter-intuitively, hubs may in fact appear *less* connected than non-hub regions due to this mixing of incoming signals, although more sophisticated measurements of statistical dependencies may help alleviate these shortcomings [22].

Secondly, most in vivo functional connectivity studies use BOLD FMRI, a signal that relates in a complex way to changes in blood flow and oxygen metabolism, which in turn have an even more complex relationship to neuronal activity [54]. Functional connectivity can also be severely affected by physiological noise and subject motion which can induce statistical dependencies that do not reflect actual connectivity [55].

Functional connectivity approaches also face major challenges in quantitative interpretability. Statistical dependencies in brain activity between regions are by no means a simple reflection of the degree of connectivity. For instance, a recent experiment performed in macaque monkeys examined the causal effect of callosotomy on functional connectivity [56]. Unsurprisingly, sectioning the commissures had a profound effect and dramatically reduced inter-hemispheric functional connectivity. However, in one monkey where the anterior commissure was preserved, the degree of inter-hemispheric functional connectivity was indistinguishable from that of control monkeys with no lesion at all. This is in spite of the anterior commissure only connecting (anatomically) restricted areas such as the amygdaloid complex and the temporal lobes [57]. Incidentally, the fact that an intact anterior commissure is sufficient to preserve wide inter-hemispheric connectivity may help explain earlier observations of intact bilateral functional connectivity in patients with agenesis of the corpus callosum [58].

Comparing connectivity inferences with functional MRI to tractography highlights the relative advantages of each technique. With tractography, we can infer which white matter pathways are carrying the connections. On the other hand, functional MRI

connectivity does not have as overt a gyral bias as tractography (although some signal-to-noise bias might be expected given known relationships between cortical thickness and folding). Another advantage of using functional measurements is the potential for assessing laminar patterns of connections (figure 1B). With increasing spatial resolution offered by ultra-high-field (7T) scanners, there is already evidence for laminar-specific functional measurements, which can potentially be used to distinguish connectivity patterns associated with different cortical layers [59]. In contrast, diffusion MRI tractography cannot reveal laminar-specific connections regardless of the spatial resolution.

Whilst it is clear that inferring structural connections from either tractography or functional connectivity has shortcomings, these limitations can manifest in very different ways. Consider the pathways linking the motor cortex and the contralateral cerebellum. These are well documented pathways (figure 3A) that in nonhuman primates follow a descending route via the brainstem and an ascending route with relays in the dentate nucleus and the thalamus. As shown previously in [60, 61], functional connectivity of the motor cortex and cerebellum correctly recovers the somatotopic organisation of these connections (figure 3B). However, only the hand representation (red) shows connections predominantly to the contralateral cerebellar hemisphere. Both the face and foot area display bilateral connectivity of roughly similar magnitude. This overall pattern presumably reflects the contributions of indirect as well as direct pathways, including differences in functional connectivity between the two cerebral hemispheres along the body map [61]. As discussed above, the difficulty in dissociating direct and indirect connections can in many ways affect the interpretability of the results.

Diffusion tractography reveals evidence for separate thalamic and brainstem routes between motor cortex and cerebellum, but fails to reveal the decussation to the correct contralateral hemisphere (figure 3C). This is due to both the complex geometry of white matter at the decussation and to the rules that tractography algorithms tend to adopt. At the decussation, cerebro-cerebellar pathways make a sharp turn to the opposite hemisphere right where pathways from each hemisphere cross each other [62]. Current algorithms for estimating crossing fibres in white matter can accurately represent the local orientations associated with these connections, however tractography algorithms use rules that do not favour sharp turns. At a crossing fibre voxel, tractography algorithms select the fibre orientation most aligned with the direction of the incoming pathway. Whilst one can envision modifications of this rule that would enable cerebellar decussation, these changes are not appropriate routinely throughout the white matter, and may cause many false positive connections that jump between different white matter tracts, whilst at the same time failing to recover long pathways that travel through complex white matter crossings. On the other hand, it is possible that some of the aforementioned complexity of cortico-cerebellar connectivity reflects axonal branching at near-right-angles along white matter bundles. If so, significant adaptations of tractography algorithms might be necessary to capture such complexity.

By contrast with the cerebellar pathways example, figure 4A-C shows a case where three different *in vivo* techniques converge, revealing the same pattern of connectivity between regions of the parietal and frontal cortices, connected via the dorsal branch of the superior longitudinal fasciculus. The same connection was found in macaques using three corresponding techniques (see figure 4D-F and [63]).

To summarise, functional connectivity can be used to infer structural long-range connections. Like tractography, however, functional connectivity is also indirect and difficult to interpret quantitatively. An interesting approach to combine the two modalities is emerging [64]. By using computational models of brain networks, one can link brain structural connectivity to network activity and functional connectivity *in humans*. This approach may perhaps allow us to get a better handle on the accuracy of tractography measures on the one hand, and on how functional connectivity relates to network structure on the other. The availability of notably high-quality diffusion imaging, resting-state fMRI, and task-fMRI data from a large number of healthy adults in the Human Connectome Project (HCP) [65] provides an excellent substrate for exploring these issues in detail.

3.3. The need for white matter organisation

Returning to the routes of connections estimated with tractography, we can use some of the quantitative information gleaned from classical neuroanatomy to highlight an important but not usually explicit assumption underlying the interpretation of all tractography results. A condition for the organisation of white matter bundles is proposed, and we postulate that connections that do not fulfil this condition may be largely invisible to tractography.

Consider intra-hemispheric, association axons, which represent the majority of white matter connections in humans. The number of such axons in humans has been estimated to be of the order of $6 \cdot 10^9$ axons per hemisphere [1]. Given that the surface area of the human cortex is on average 10^5 mm^2 per hemisphere [66], we estimate that each square millimetre of cortex on average sends/receives $6 \cdot 10^4$ axons going to/coming from other cortical regions of the same hemisphere. These sixty thousand axons are distributed into short, middle, and long range projections. Recent, quantitative tracer data in the macaque reported that the number of cortico-cortical axons decreases exponentially with the distance between the source and target area [10]. For instance, whilst 40% of these axons project to within 10mm, fewer than 1% travel a distance of 35mm or more (the distance between e.g. primary visual cortex and anterior temporal lobe in macaques). Considering that a white matter voxel at high spatial resolution may contain $3 \cdot 10^5$ axons, this means that the contribution of axons projecting from a distant square millimetre of cortex may be about 0.2% of a white matter voxel near the target, i.e. each long-range connection may contribute very little to the measured signal in each voxel.

The above numbers are of course very approximate. Retrograde tracing can be used to count cell bodies, but translating these estimates into axon numbers is not straightforward. Also, some of the long association pathways may be significantly stronger than others and the contribution of a square millimetre of cortex may be much larger for these connections. Furthermore, this analysis does not necessarily apply to commissures and projection pathways (although the number of such connections is even smaller, 1-2000 axons per mm^2 of cortex [1]). Nonetheless, we are confronted with the fact that, on average, long range associative connections may have a very small contribution to the signal in a voxel, at least from the point of view of the "dense" connectome [65], i.e. if we do not summarise the connections over large regions spanning many square millimetres.

How, then, can tractography ever work to identify long range connections? The key to successfully detecting the routes of connections via tractography is white matter organisation. Given the spatial scale of in vivo techniques relative to that of axons, white matter axons are bound to intermix. In order to be able to differentiate the trajectories of intermixing axon bundles, tractography algorithms must be able to make inferences at sub-voxel accuracy while reflecting the diversity of gray matter origin/termination. This may be possible if the intermixing is non-random, but spatially organised, such that the axonal inputs/outputs for neighbouring cortical regions tend to occupy neighbouring pieces of white matter. If such spatial organisation is not present or not measurable, then a different postulate of (statistical) white matter organisation may be necessary for tracking long-range connections. This postulate may be stated thusly: "in a given white matter voxel, the probability distribution of axonal terminations is independent of their origin". In other words, if axons share a bundle and are indistinguishable (in terms of location and direction) from each other within the bundle, then they must connect to the same regions and in the same proportions regardless of where they originated from so that tractography becomes algorithmically tractable.

This postulate of organisation, to the extent that it is respected, may alleviate the intermixing issues due to scale differences between axons and voxels. However, the degree to which white matter is organised along such lines is not known. One can hope that spatial organisation may naturally arise during brain development, when axons are thought to be guided by chemical gradients [67] that in turn may induce topographic continuity in the connections, as suggested in [68]. The extent to which this is true throughout white matter will ultimately determine the success or failure of tracking long-range connections all the way to their cortical termination, i.e. generating accurate large-scale structural connectomes.

3.4. Evidence for organisation

Although a complete picture of white matter organisation, particularly for the human brain, is not currently available, evidence in favour of this hypothesis of spatial organisation is accumulating. The plethora of tracer studies in animals are a key source of anatomical information. Of particular interest are tracer studies that report the entire trajectories of axonal projections from source to target regions, and can therefore inform us on organisation. For instance, projections from the macaque orbitofrontal cortex appear to follow a spatial organisation dictated by the medial-lateral position of their origin [69]. The same organisation was found using diffusion tractography in post-mortem macaque brains and in humans in vivo [26]. Conversely, the degree of accuracy of tractography results is also indirect evidence for white matter organisation. Direct comparisons between tractography and autoradiographic tracing of several association pathways in macaques reveal very good qualitative agreement between the techniques [70], suggesting that tractography can reconstruct and distinguish long-range association pathways.

In humans, where the organisation of connections is mostly unknown, *indirect* evidence for the accuracy of tractography can be gleaned from the numerous studies that relate tractography results to function and behaviour. If all tractography results are artefactual, then they need not bear relationships to measurements of function that have independent sources of noise. In the following section, we give an overview that illustrates the power of relating structure and function in-vivo. We also present

examples of approaches that capitalize on the strengths of in vivo methods to obtain information that is not available by other means.

4. Unique potential and applications of in vivo tools

In vivo imaging methods potentially provide a powerful toolbox. Many connections and many subjects can be studied in health or disease [71]. The ability to relate connections to behaviour and genetics can provide new insights into variability across the population [72] and has formed the basis of large-scale coordinated efforts (such as the Human Connectome Project [65] and the ENIGMA consortium [73]). Longitudinal studies open the possibility to explore developmental and aging processes [74], while latest technology makes neonatal/foetal imaging more accessible [75]. Comparative studies across species using imaging methods can also help bridge the gap between invasive animal studies and non-invasive human studies and translate findings from animals to humans (or vice versa). In the overview that follows we give some representative examples of these applications, focusing on demonstrations that provide indirect evidence of the accuracy of in vivo methods.

4.1. Relating structure to function and behaviour

Identifying boundaries of functionally-distinct brain areas using tractography-estimated connections provides an indirect means of assessing the utility of tractography. It also provides indirect evidence for white matter organisation that is necessary for long-range tracking to succeed.

Tractography has been used to identify locations within the thalamus that preferentially connect to particular cortical regions [76]. When these “connectional localisers” are compared against meta-analyses of task fMRI activations [77], or resting-state connectivity in the same subjects [78], there is striking correspondence. More direct evidence has been reported in individuals undergoing neurosurgery for epilepsy who had depth electrodes in thalamus and a grid of electrodes in cortex [79]. Somatosensory evoked potentials (SEP) recorded using electrodes covering an extended area of the thalamus revealed a restricted area of thalamus where the evoked potentials showed a phase reversal, which indicates the source of the SEPs. The phase reversal occurred at the precise location where the thalamus is preferentially connected to the somatosensory cortex according to in vivo tractography in the same patients. A notable application of such in vivo functional localisation is deep brain stimulation of sub-cortical structures for the treatment of depression [80, 81], chronic pain [82], or tremors in Parkinson's disease [83].

Detailed predictions can also be made about functional boundaries in cortex. If extrinsic connections of a grey matter region constitute a signature of its functional role [2], by searching for cortical locations that exhibit sharp transitions in connections, it should be possible to identify functional boundaries [46, 84]. Indeed, such approaches have now been applied across a broad range of cortical regions [85], and have been shown to predict boundaries between fMRI task activations [84], resting functional connectivity [86], and areas defined cytoarchitectonically [87]. It is not clear, though, to what extent these parcellation techniques are affected by issues such as the gyral bias. Determining the boundary between cortical regions using

tractography should ideally be augmented with evidence from other modalities, for example using the aforementioned multimodal HCP datasets.

Most recently, techniques have emerged to explore the relationship between structural and functional anatomy in even greater detail. Indeed, it is argued that it is possible to make detailed predictions of functional activation patterns across a large portion of the temporal lobe on the basis of the connectional anatomy of the region, measured by tractography [88]. That such an approach, based on long distance projections, is predictive of functional measurements, suggests that these projections exhibit favourable organisation.

Finally, there is growing evidence that (semi-)quantitative measurements from tractography can predict regional functional and cross-subject behavioural variation. A good example is provided by a network of cortical and subcortical regions known to be involved in inhibitory control of actions [89], and which was studied by several independent laboratories using human *in vivo* connectomics. This network includes the inferior frontal gyrus, medial prefrontal cortex, and subthalamic nucleus. Connections between these regions can be reproducibly mapped using tractography [90]. Notably, variations between individuals in their ability to inhibit actions relate to the strength of connectivity between the sub-thalamic nucleus and medial prefrontal cortex [91]. Similarly, cross-subject variations in callosal projections between bilateral supplemental motor areas are correlated with differences in bimanual coordination [92], and variations in striatal projections to limbic cortical and subcortical structures correlate with differences in subjects' propensities for reward and novelty seeking behaviours [93].

The observed correlation between structural connections and behaviour may be mediated by functional responses, and the *in-vivo* nature of tractography allows direct tests of this hypothesis. For example, synchronous oscillations between hippocampus and frontal cortex are known to be correlated with memory performance in both rodents [94] and humans [95]. Using tractography, it is possible to measure the anatomical projections associated with this synchrony. Indeed, subjects with stronger hippocampal prefrontal projections exhibit slower synchronous oscillations, and better long-term memory encoding [96]. Similar predictions can be tested about more focal projections between small subcortical nuclei. Connections from the dopaminergic midbrain to ventral striatum vary between subjects and have substantial consequences on ventral striatal functional responses. The BOLD signal in ventral striatum (VS) is known to code for a reward prediction error [97, 98], and it has been suggested that this signal depends on dopaminergic input, as dopamine cells are famous for a similar pattern of reward coding [99]. Across subjects, the extent to which such prediction error coding can be measured in the ventral striatum is predicted by the strength of connection (measured by diffusion tractography) between VS and the dopaminergic mid-brain [100]. Notably, the same change to the VS functional response can be induced by delivery of Dopamine agonist L-DOPA [100]. Hence the VS signal appears more similar to the dopaminergic cellular response both in subjects treated with a dopamine agonist, and in subjects who have a larger mid-brain-VS projection.

4.2. Learning and augmenting organisational principles

In animal models, it is possible to combine diffusion MRI tractography with tract tracing in order to validate the non-invasive tools [26, 101, 102]. Such validation work is important for developing new algorithms and models that are a better fit to known anatomy. The combination of tracing and tractography is not only valuable for validation, but can also be used to ask questions that augment the findings of tracers, given that the latter can only inform us, albeit with great precision, about a small set of connections at once.

In [26], we used tracers and tractography of the macaque ventral prefrontal cortex (vPFC) to ask whether a set of rules suggested by the tracer results [69] generalise to the entire vPFC. Injections of tracers into three locations of vPFC suggest a pattern of organisation for these pathways: the medial-lateral position of the injection sites in the vPFC dictate the relative position of the corresponding pathways when they reach the corpus callosum and internal capsule. What the tracer data did not address is whether this pattern is generalizable to the entire vPFC, or whether a similar rule applies in humans.

Diffusion tractography is well suited for addressing both questions. Figure 5 shows the results, which confirm that the medial-lateral organisation not only generalises across the vPFC, but also across species. Similarly, resting-state fMRI data have been recently used to map the organisation of connections between ventral prefrontal cortex and parietal cortex in humans, and confirmed the rostro-caudal arrangement found in tracer studies of macaques [103].

These approaches illustrate the power of non-invasive tools in combination with restricted, but accurate information from the more reliable invasive techniques. By imaging connections in the entire brain, as opposed to a few connections at a time, we have the ability to analyse *spatial* organisation.

4.3. Comparative anatomy across species

The vast amount of electrophysiological and neuroanatomical data from nonhuman primate studies provide a framework for interpreting human data. But comparative studies of connective anatomy between species are needed to determine the extent to which inferences from monkeys can inform us about human brains. For instance, an important set of questions is how to determine homologue areas across species. This was traditionally achieved using combinations of macroscopic morphological landmarks and cytoarchitectonic markers. For instance, thirteen subregions of the orbito-frontal cortex have been reported in macaques and humans using multiple architectonic criteria, with precise one-to-one correspondence [104, 105], and a putative homologue of human's Broca's area has also been reported in the macaque brain [106].

Connections have also been used to provide signatures for brain areas that can be used to determine cross-species correspondence. Interestingly, this type of approach has been formulated in both directions, by transferring knowledge from the macaque to humans, and vice versa. A study of the parietal cortex in macaques and humans [107] identified three sub-regions surrounding the intra-parietal sulcus with well documented connection patterns from anatomical tracer studies. These three parietal regions can be distinguished by a sub-set of their anatomical connections (see figure

6). In particular, three well separated targets can be used as a signature for the parietal areas, providing candidate homologue areas.

A more recent comparative study used the same logic to infer homologies in the opposite direction [108]. This study was interested in a region of the temporo-parietal junction (TPJ) that fMRI studies in humans implicated in theory of mind [109]. The question of whether a similar region exists in macaques cannot be addressed by looking for similar task-related activations in nonhuman primates because there is no evidence that nonhuman primates engage in theory-of-mind tasks in the same manner as humans. Using functional connectivity fingerprinting, the authors identified a set of target regions in humans that (i) uniquely determine TPJ's connectivity, and (ii) can easily be identified in the macaque. The study found a region of the superior temporal sulcus in the macaque that has a connectional pattern resembling that of human TPJ. Interestingly, the same area was previously found to be morphologically correlated with the macaque social network size [110].

This idea of cross-species localization using connectivity is a powerful one. It has already been used to propose homologies for the entire prefrontal cortex [111], lateral parietal cortex [112, 113], and medial parietal cortex [114].

5. Conclusion

Tracer studies point to both structured and complex arrangements of long-range connections, which in-vivo techniques are hoping to be able to detect. Current MRI-based approaches are powerful but have several shortcomings due to their indirect nature and low resolution. The availability of these non-invasive tools has greatly broadened the spectrum of neuroscientific questions that we can now investigate. These tools must be used with great care, and combined whenever possible in order to alleviate their respective caveats. They should not, however, be considered as "poor man's" tracers. The ability to image the whole brain and combine functional and behavioural measurements with connectivity is a real asset that tracer techniques cannot replace.

Competing financial interests: **NO**, I declare that the authors have no competing interests as defined by Nature Publishing Group, or other interests that might be perceived to influence the results and/or discussion reported in this paper.

Figure Captions

Figure 1.

Examples of complex white matter organisation in monkey brains. A. topographic organisation of afferent ipsilateral and contralateral connections to the principal sulcus in the macaque brain, showing columnar interdigitation. Typical 1mm and 0.5mm isotropic voxels are shown as dashed boxes, indicating the resolution needed to reveal such macro-scale organisation (modified from [9]). B. Laminar pattern of efferent connections from visual area V2. Cells connecting to V1 sit primarily in superficial and deep layers, whereas connecting to V4 originate from layer 2/3. No labelled cells are found in layer 4 (modified from [115]). C top. Estimate of the

number of efferent ipsilateral association axons from 1 mm² of cortex as a function of the distance between source and target regions. C bottom. Estimate of the number of axons from 1 mm² of cortex forming different categories of fibre tracts. Estimates are based on [1] and data from <http://core-nets.org>).

Figure 2.

Diffusion MRI and tractography. (A) A coronal section through a human brain (left) and estimated fibre orientations from diffusion MRI data (right). Voxel-wise fibres are colour-coded according to their orientation (red=left-right, green=anterior-posterior, blue=ventral-dorsal). Major fibre bundles can be visualised on the orientation maps (cing=cingulum bundle, cc=corpus callosum, cst=cortico-spinal tract, slf=superior longitudinal fasciculus). Note also the many voxels with multiple orientation estimates (crossing fibres) allowing the major bundles to cross each other. Tractography algorithms use this type of local orientation estimates to infer long trajectories of white matter bundles. Data from the Human Connectome Project [65, 116].

(B) Probabilistic tractography of the corpus callosum pathways consists in constructing a spatial histogram that represents the likelihood that streamlines, through the diffusion field, pass through any voxel of the brain. The scenario of a single fibre orientation within a voxel is not always representative of the underlying anatomy. Crossings of fibre bundles are very common in white matter. The figure shows probability maps arising from the body of the corpus callosum when modelling multiple vs single fibre orientations. Ignoring fibre crossings gives rise to many false negatives (the lateral callosal projections are missing), but also false positives (paths merging with the internal capsule).

(C) Ambiguities in modelling voxel-wise fibre orientations: Four different putative voxel-wise patterns of axonal organisation can give rise to the same diffusion scatter pattern when averaged over a voxel. Top-left=bending fibres, top-right="kissing" fibres, bottom-left=inter-digitated fibres and bottom-right="touching" fibres. Simple crossing fibre modelling cannot distinguish these cases, which may lead to false positive and false negative connections. Figure reproduced with permission from [117].

Figure 3. Text-book and estimated cortico-cerebellar connections using functional and diffusion MRI. (A) Multi-synaptic efferent (blue) and afferent (red) cerebellar connections decussate at the level of the pons. (B) Resting-state functional connectivity of motor cortical regions to cerebellum (foot, hand and face area as green, red and blue). The somatotopic organisation is evident, but notice that, except for the hand area, homologue seed areas in the right and left hemisphere may yield almost identical connectivity results. (C) Diffusion MRI tractography streamlines when seeding from the hand area of the primary motor cortex. Tractography reconstructs the correct paths, but fails to decussate at the pons due to the smoothness constraints used in tracking algorithms.

Figure 4. Agreement between functional and structural connectivity in measuring connections in human and macaques. Top: Connectivity maps for area IPS3 obtained from data acquired for the Human Connectome Project [65] (average connectivity of 40 unrelated subjects). (A) Functional connectivity using correlations of resting-state fMRI timeseries. (B) Functional connectivity using correlations of activations across multiple tasks (7 tasks and 42 contrasts). (C) Structural connectivity using diffusion

MRI and probabilistic tractography. Bottom (adapted from [63]): (D) Map of voxels exhibiting BOLD correlations in spontaneous activity amongst at least 3 out of 4 regions of the oculomotor system in the anaesthetised macaque (dorsal views, AS: arcuate sulcus, CeS: central sulcus, FEF: frontal eye fields, IPS: intraparietal sulcus, LIP: lateral intraparietal area, MT: middle temporal area, SF: sylvian fissure, STS: superior temporal sulcus). (E) Activation pattern evoked by performance of a saccadic eye movement task (average of two monkeys). (F) Density of cells labelled by retrograde tracer injections into LIP (average of three monkeys).

Figure 5. Testing generic organisation principles using tractography. Injection of tracers into three locations of the macaque ventral prefrontal cortex (vPFC) reveals that the medial-lateral position of the injection sites in the vPFC dictates the relative position of the corresponding pathways within the genu of the corpus callosum. Tractography is then utilised to test and confirm that this pattern is generalizable to the entire vPFC, both in macaques and humans. The scatter plots show the positions of the centers of gravity of the vPFC seed regions plotted against the centers of gravity of the pathways. It is clear that the x-position (medial-lateral) of the seed regions correlates significantly with the z-position (ventral-dorsal) of the corpus callosum projections. The insets show subdivisions of the vPFC into 13 regions according to [105] for macaques and [104] for humans. The regions are colored in red/green/blue according to their approximate medial-dorsal positions for ease of visualization. Figure adapted from [69] and [26].

Figure 6. Finding homologue areas across species. Left: Activity in the temporo-parietal junction (TPJ) is associated with “theory of mind”, the human ability to infer thoughts and beliefs of others. Functional connectivity fingerprinting in humans reveals high correlations with the anterior and posterior cingulate areas and no interactions with the cingulate motor area and the anterior insula. A candidate homologue area can be found by searching for areas in the macaque cortex with the same connectivity profile [108]. Right: Three areas surrounding the macaque intraparietal sulcus have been found through tracer studies to have distinct connection patterns to the superior colliculus (red), parahippocampal gyrus (blue) and ventral premotor cortex (green) respectively. Similar subdivisions on the human parietal cortex can be found using tractography seeded in the parietal cortex and guided by these three macaque-inspired targets [107].

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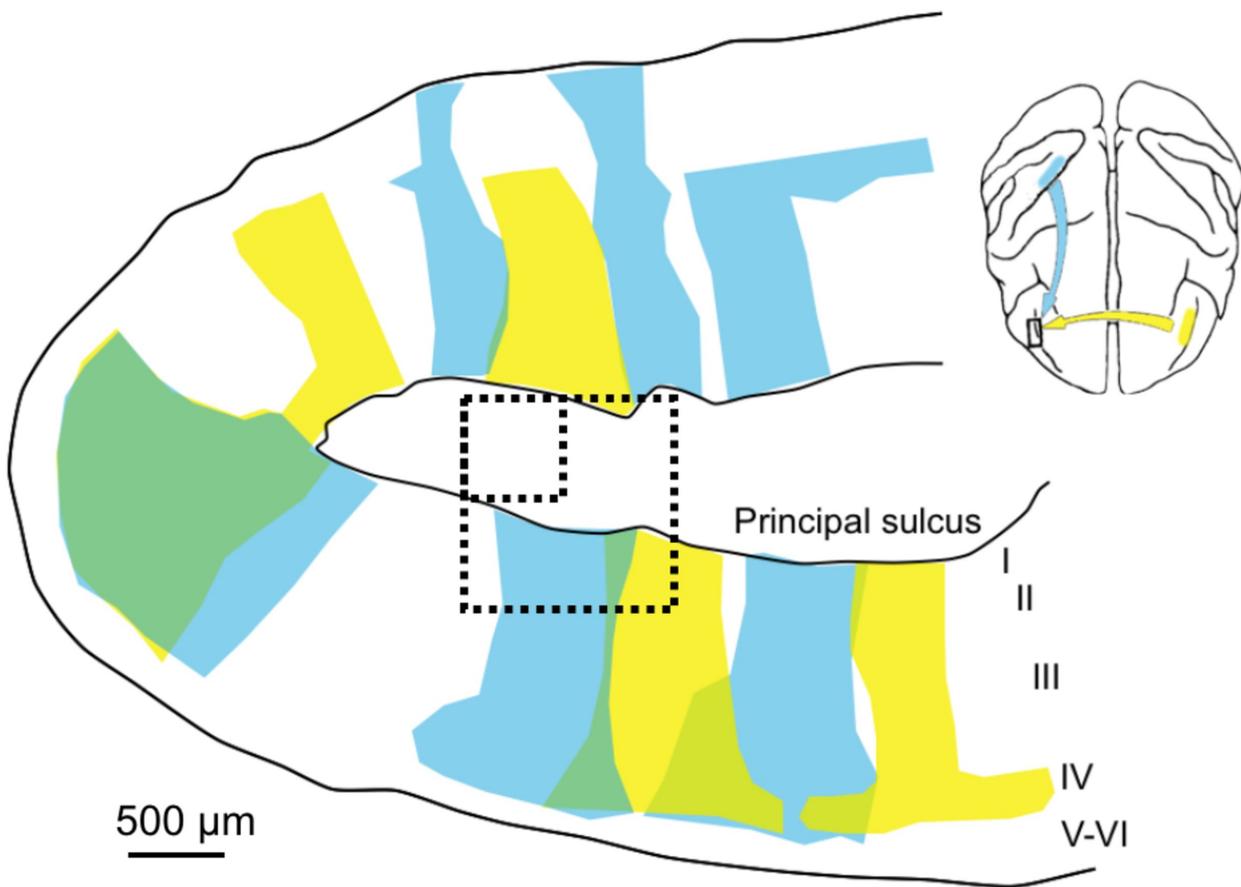
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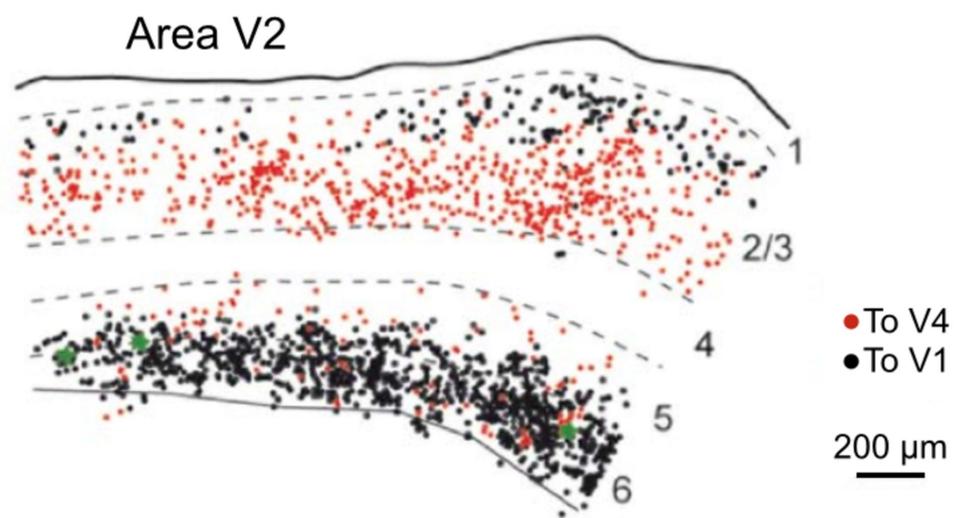
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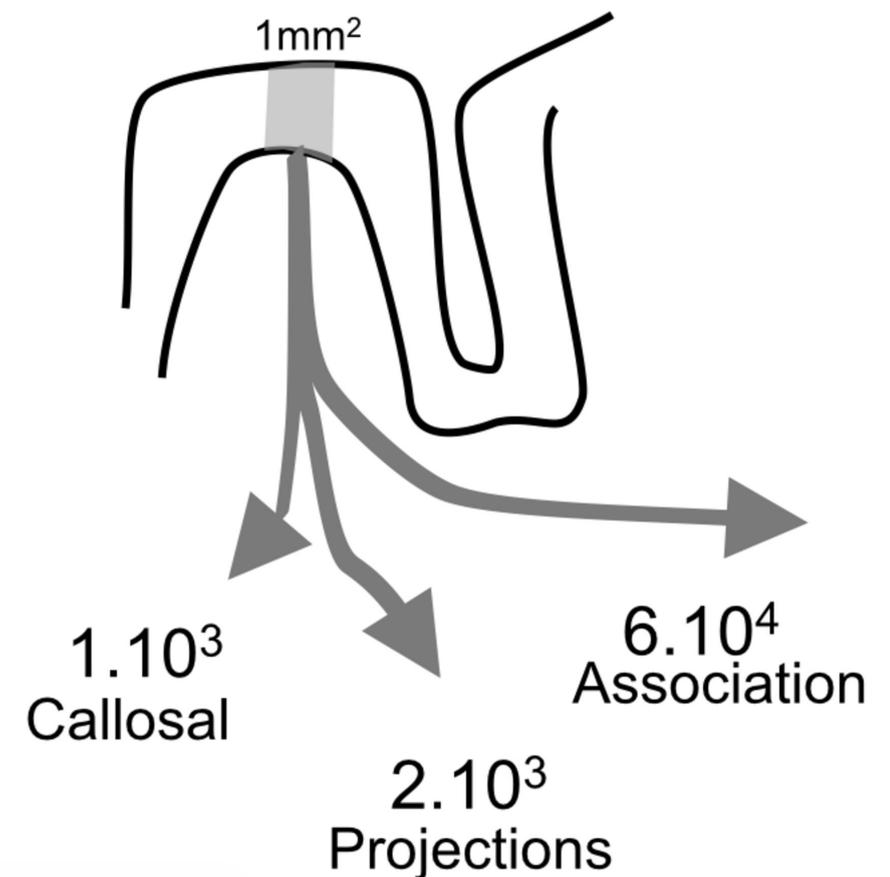
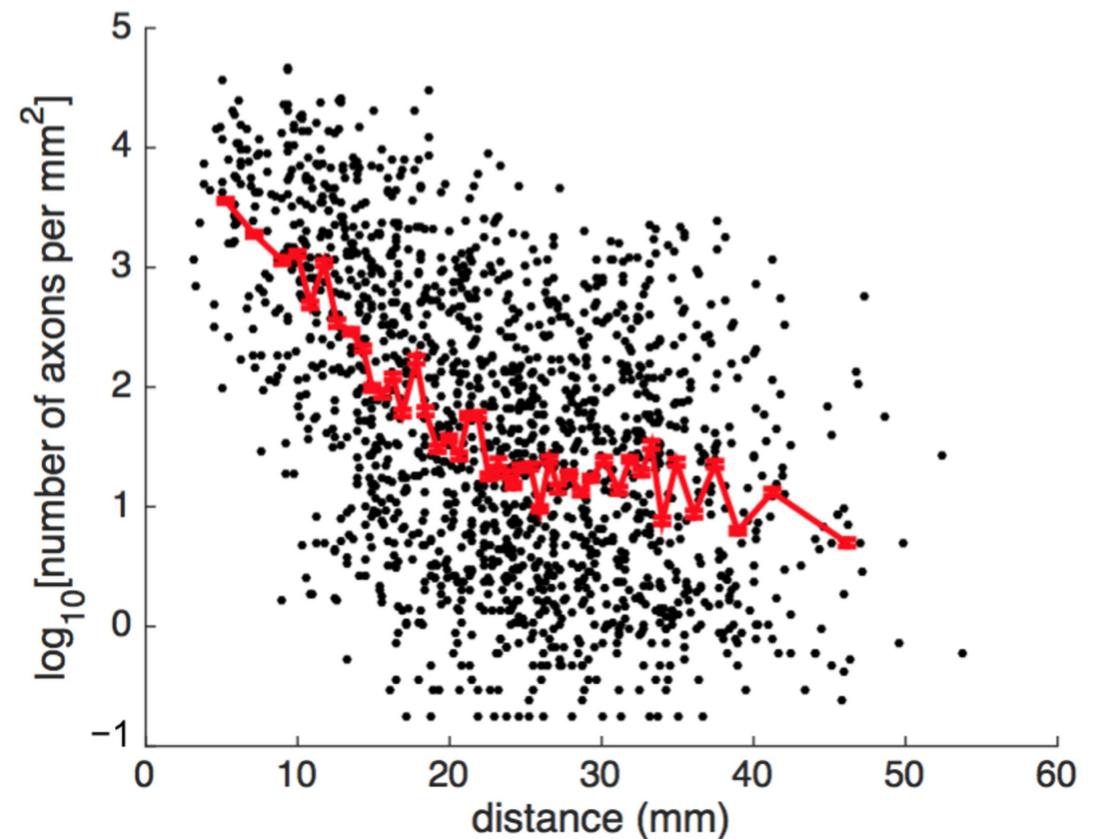
A Topographic organisation

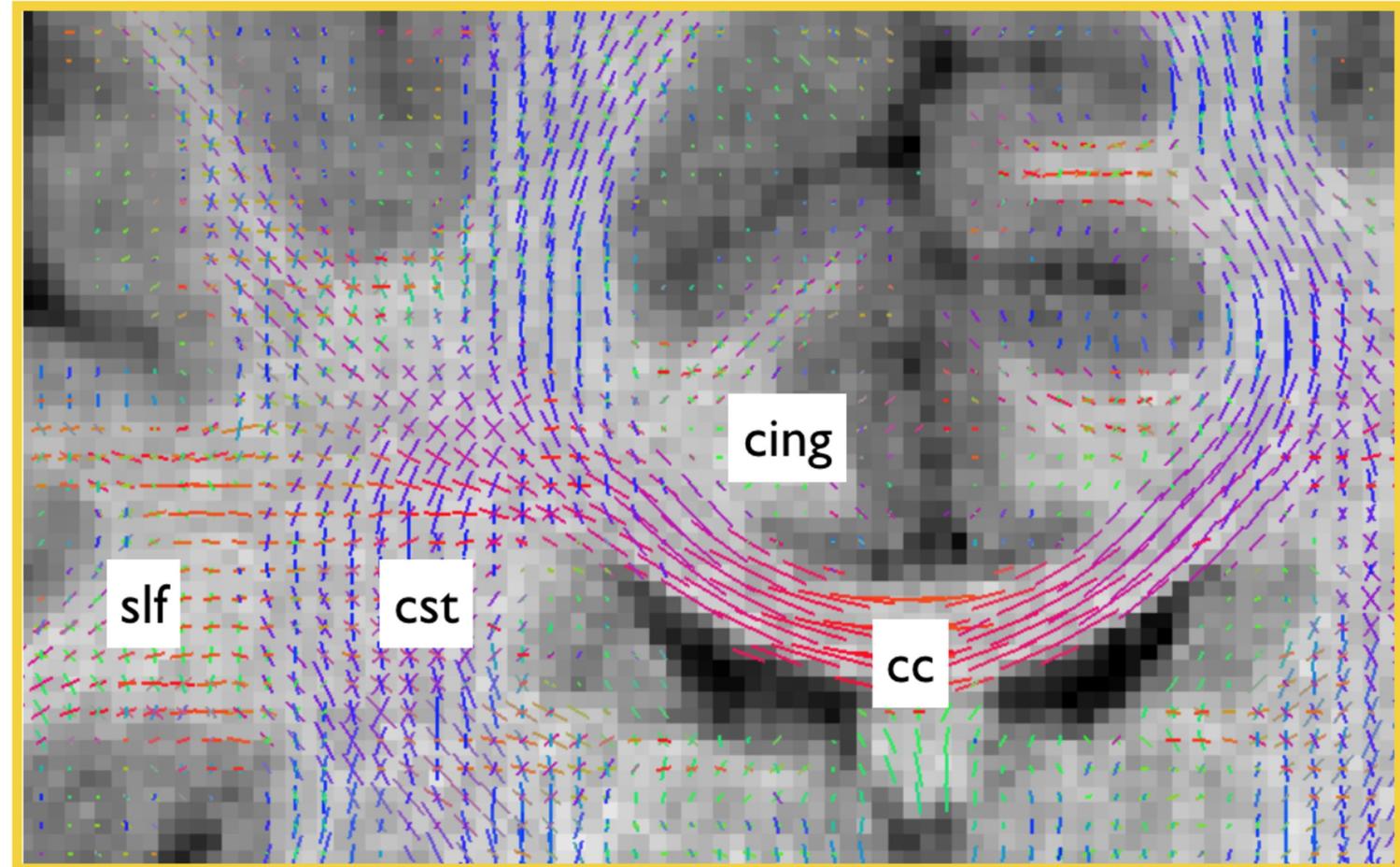
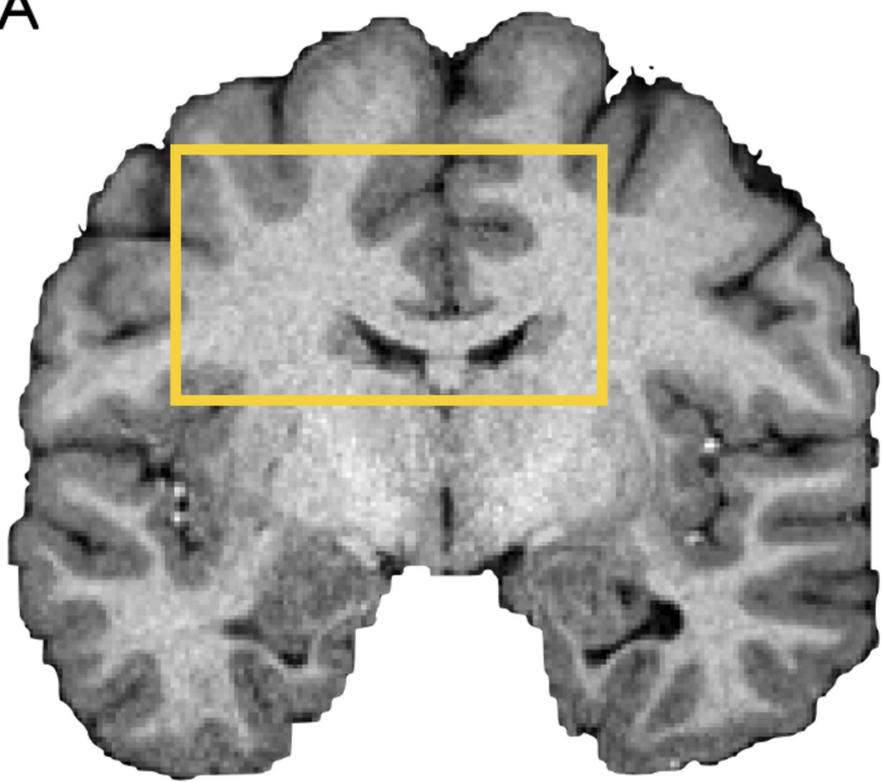
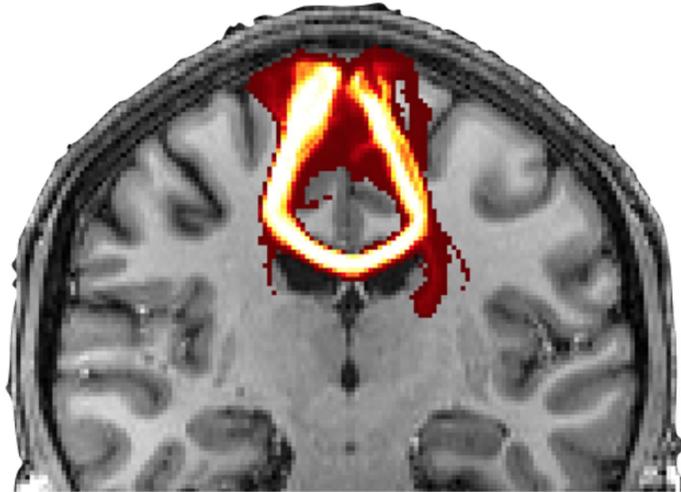
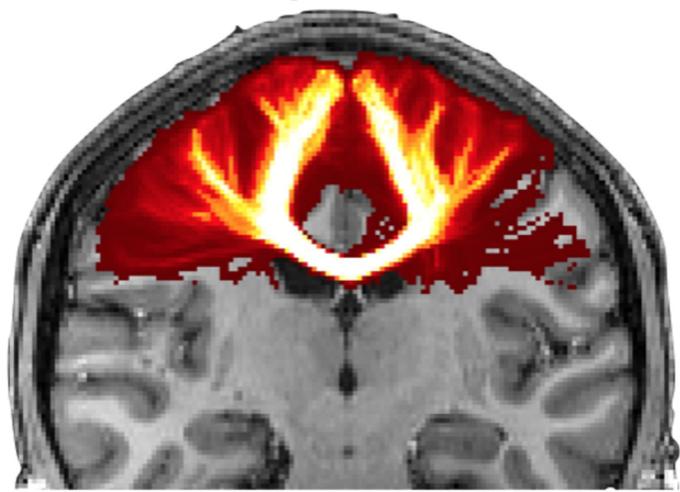


B Laminar organisation

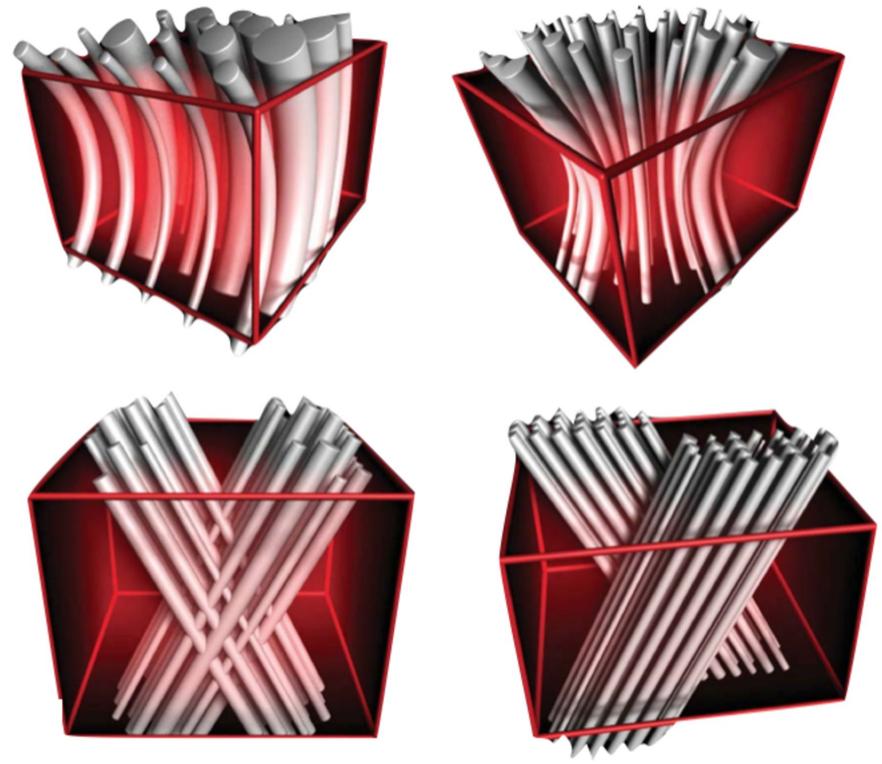


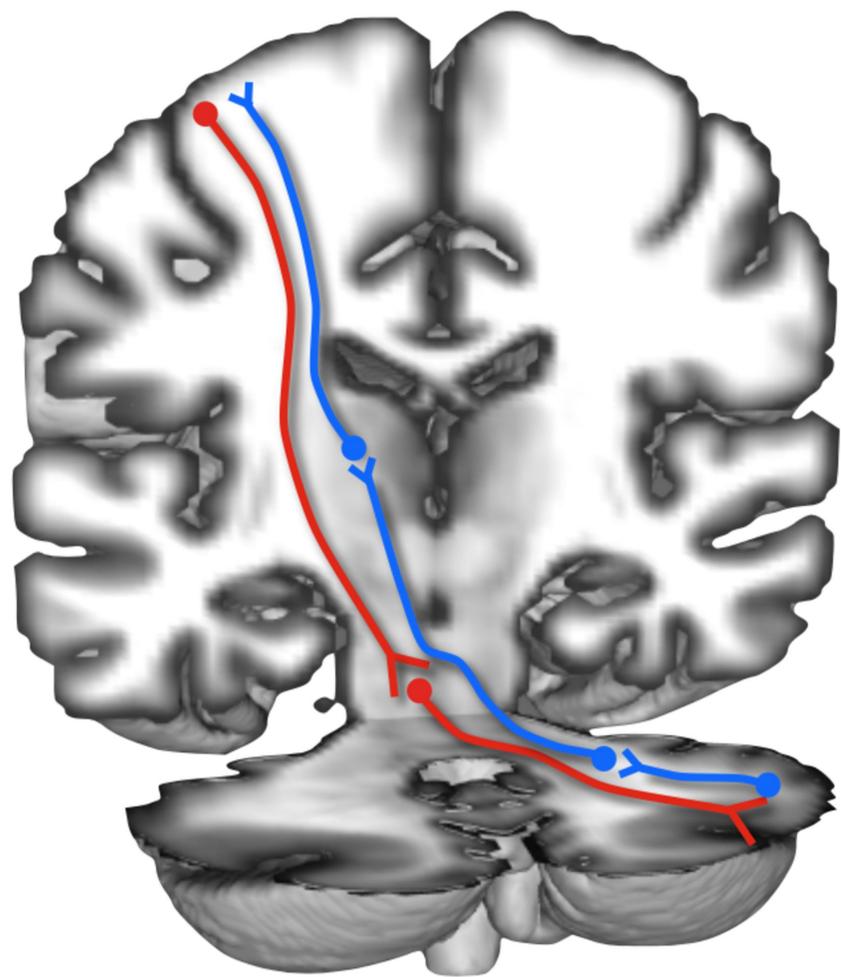
C Quantitative data



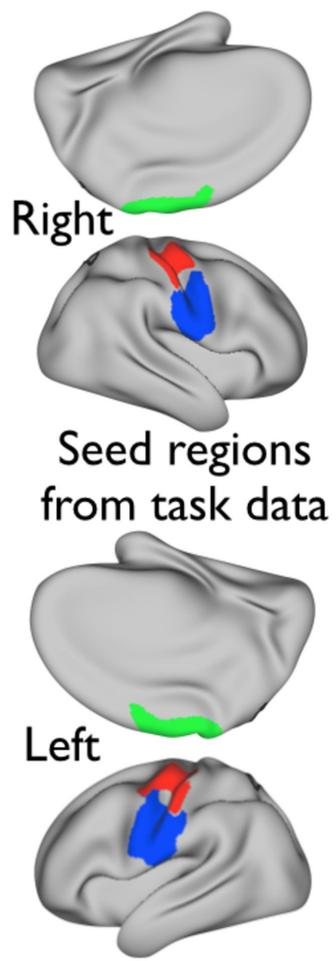
A**B****Single fibre****Multiple fibres**

Low probability  High probability

C

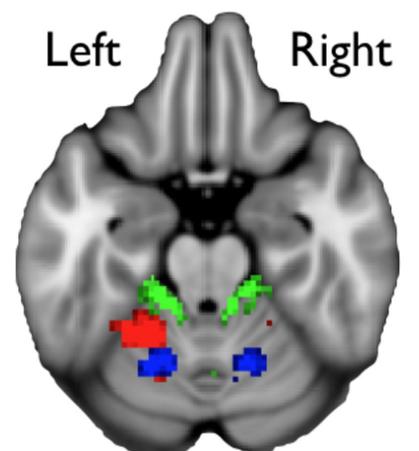


A Textbook connections

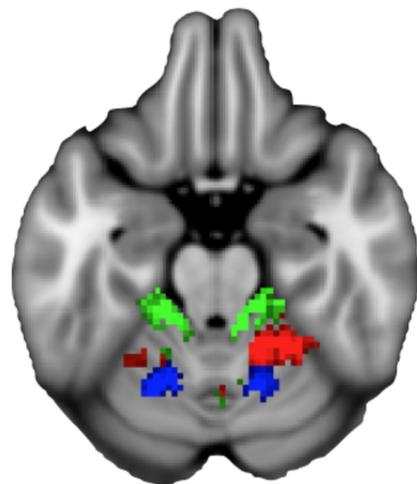


Seed regions
from task data

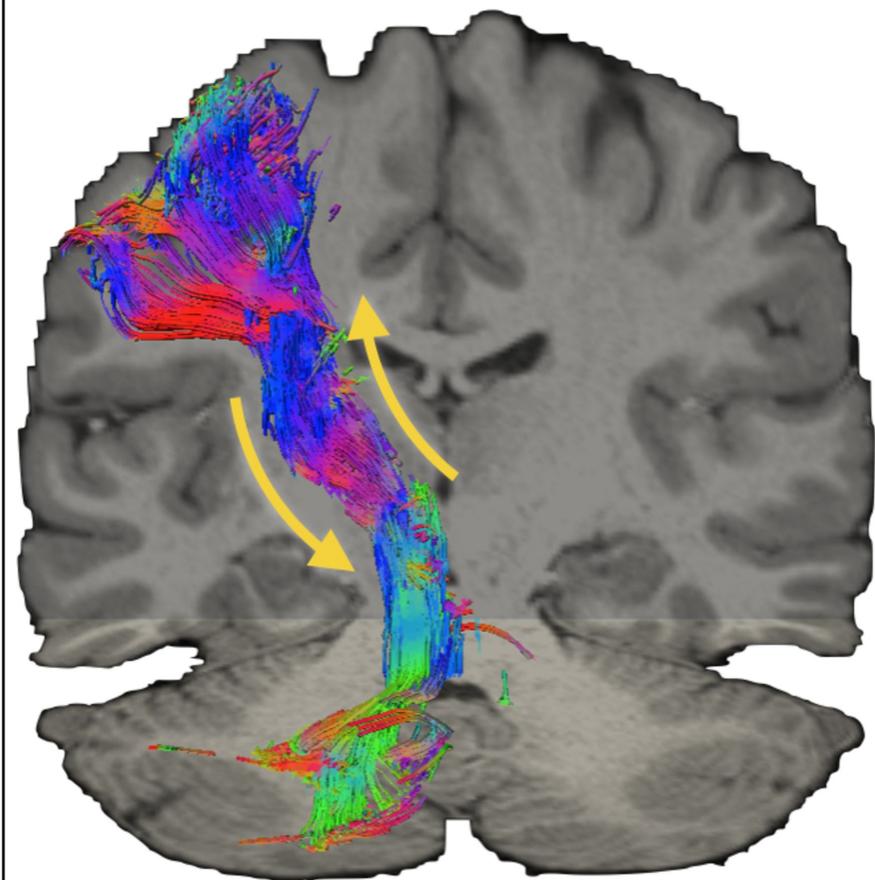
B



RFMRI connectivity

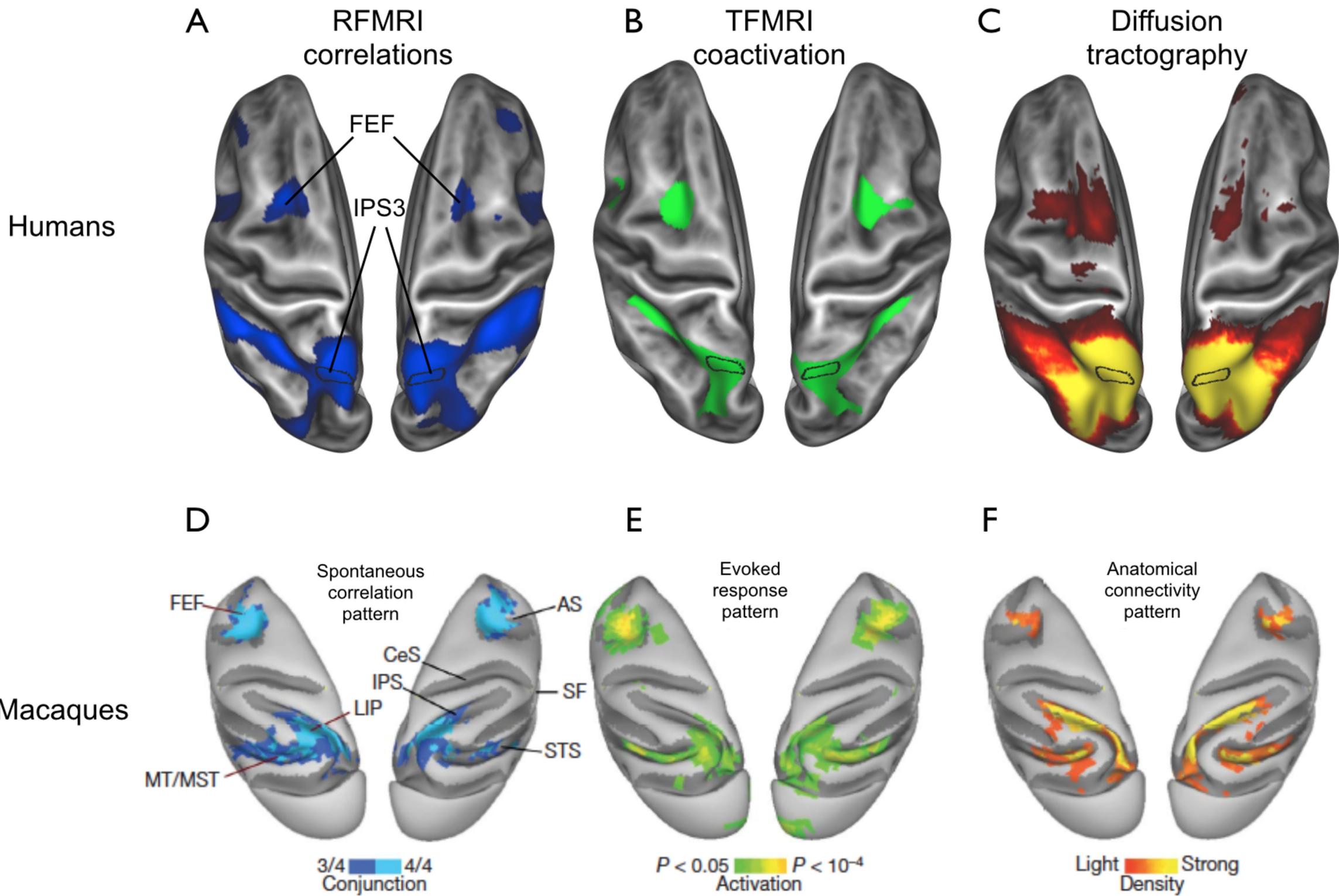


Left Right

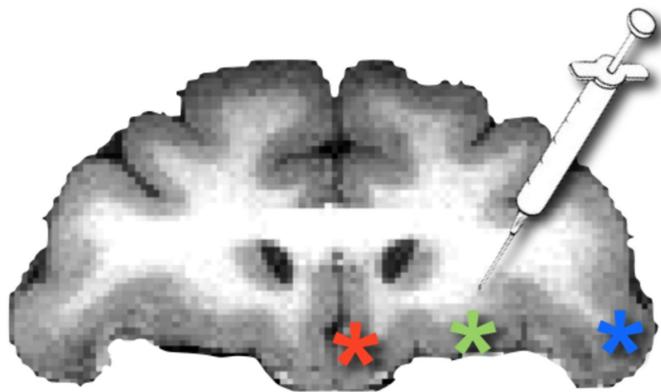


Tractography

C

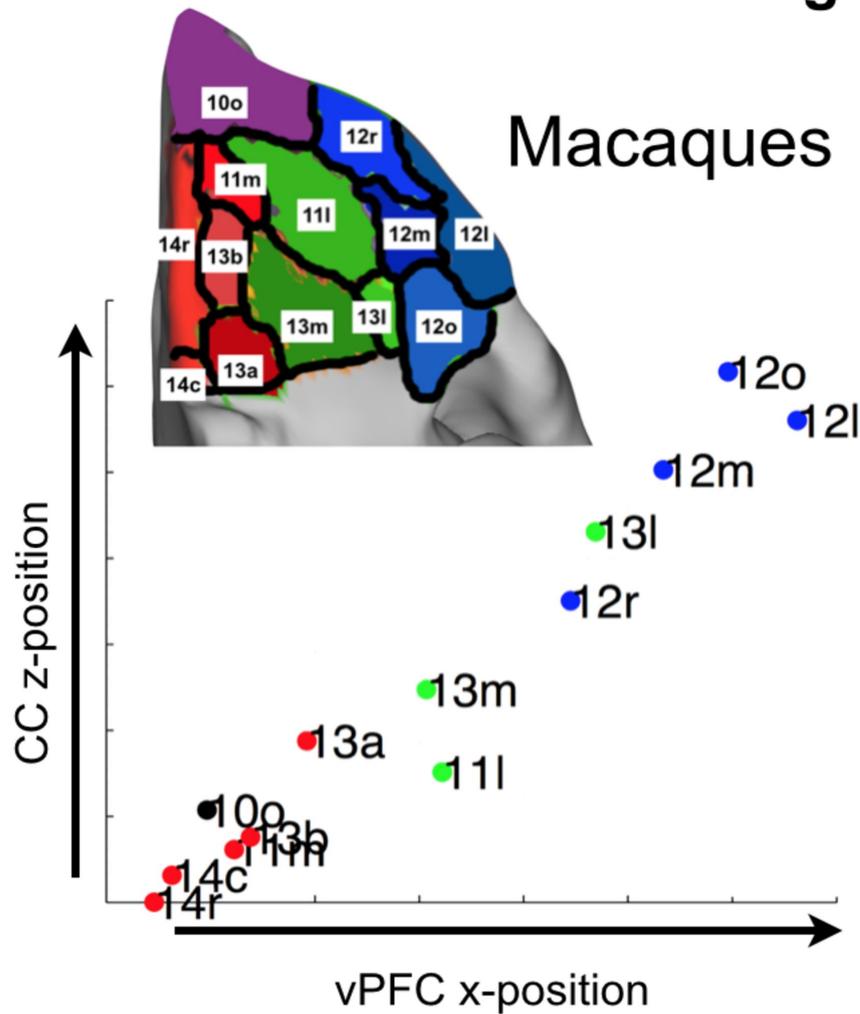


Tracing

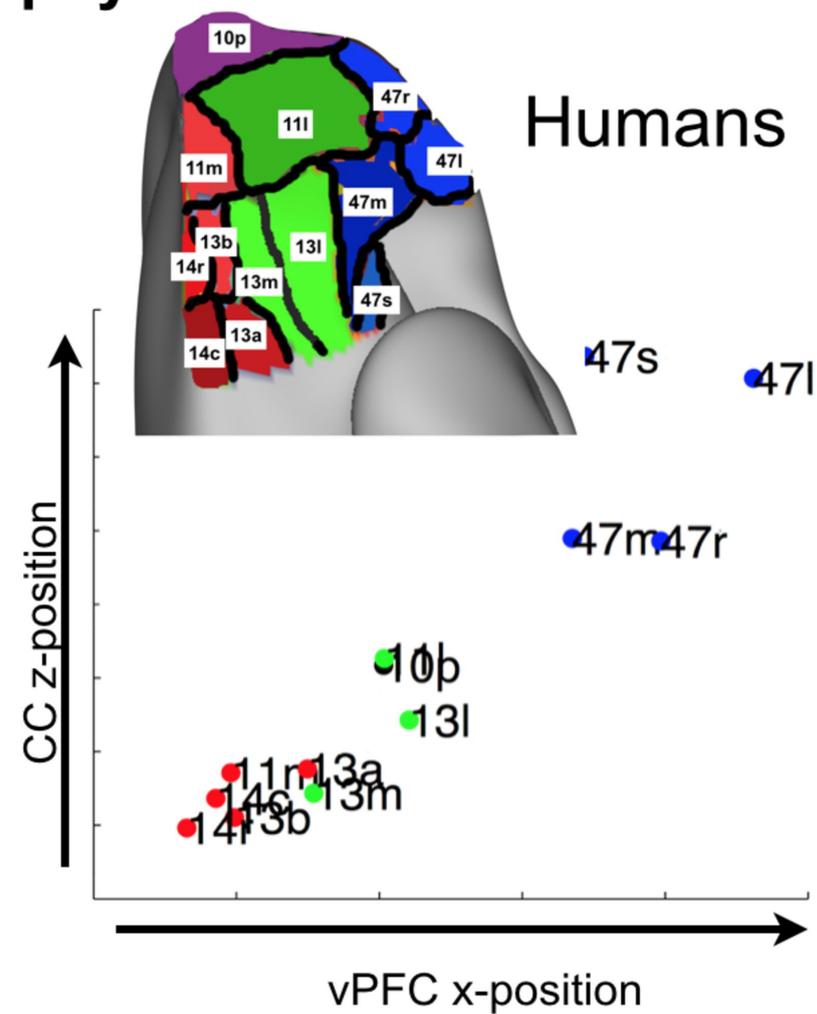


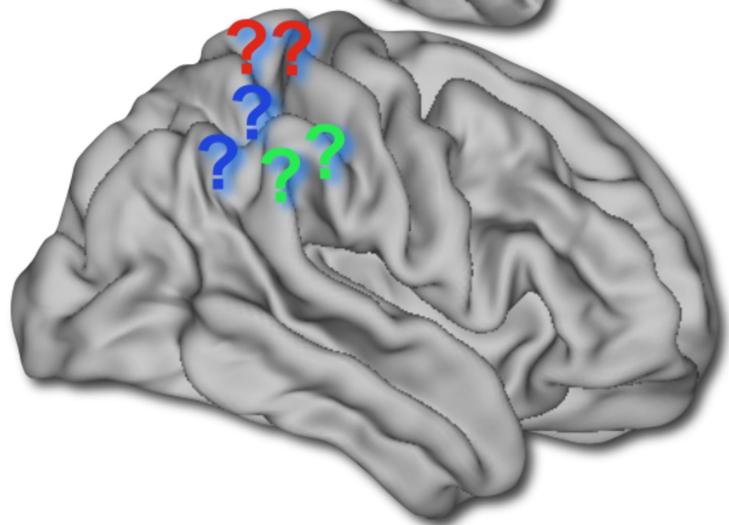
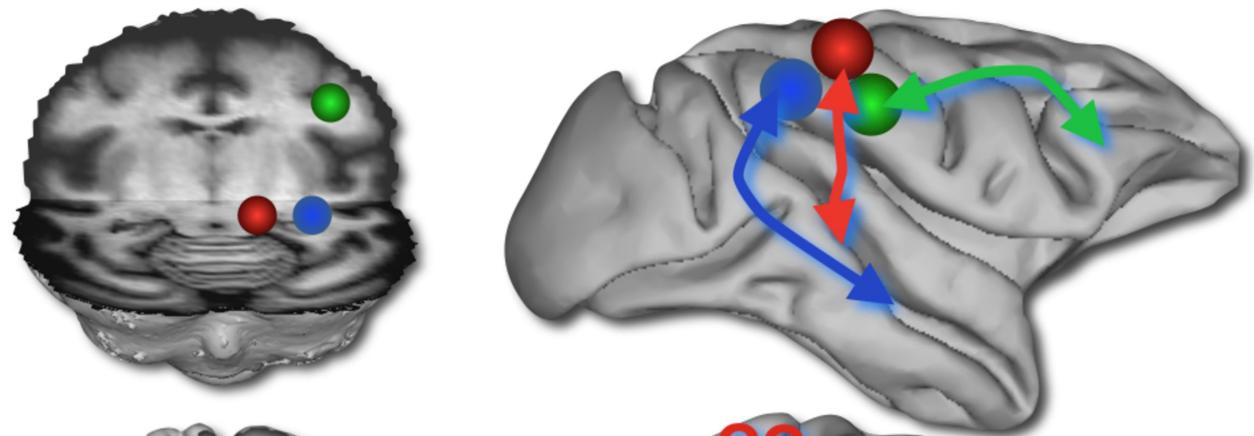
Tractography

Macaques

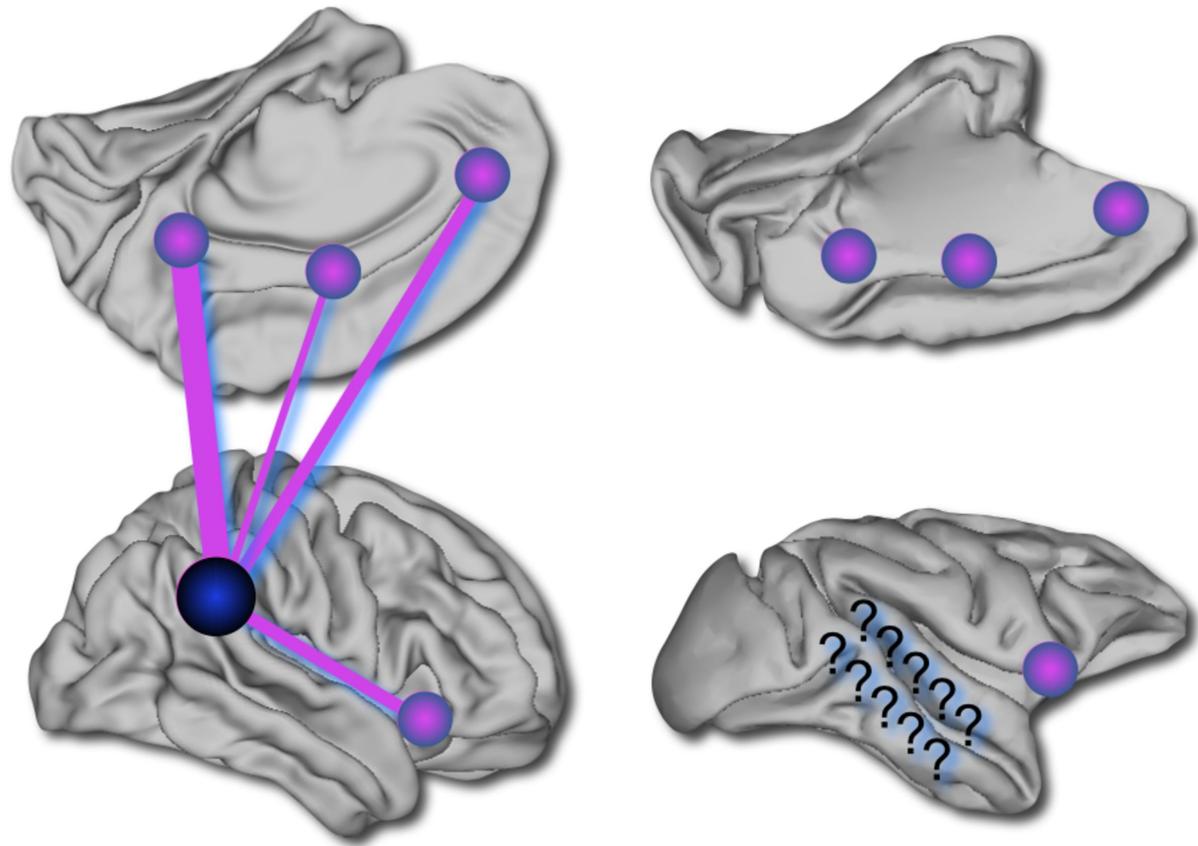


Humans





Macaque → Human



Human → Macaque