Measuring macroscopic brain connections *in vivo*

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Decades of detailed anatomical tracer studies in non-human animals point to a rich and complex organization 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.

Connections have 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 10% of the total connections in the brain[1], yet they are crucial 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 50 years. Techniques for measuring at the micro-scale (neurons, axons, synapses) are entering an industrial era, with fast automated imaging (for example, serial block-faced electron microscopy[3]) replacing labor-intensive approaches. At the macro-scale (regions, fiber 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 a great deal of 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, behavior and other types of metadata that can be related to connections.

This review highlights some of the key challenges for *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.

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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 organization 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 with subcortical and commissural pathways. This appears to be true even when restricting consideration to non-local, long-range connections. One point that is still debated is the density of the large-scale cortico-cortical network. Although some investigators report a relatively sparse network (10% according to ref. 4), others point to a more dense area-to-area connectivity matrix (66% according to ref. 5). If confirmed, such a high-density graph implies that macaque (and presumably also human) white matter contains more than 10,000 pathways with distinct origins and terminations[6]. In sharing a restricted space, these pathways must necessarily take a variety of complex trajectories in the white matter to reach their targets. These can include tightly fasciculated tracts over long distances as well as dispersion and/or branching from or into fascicles. Thus, although white matter appears to be grossly organized into a relatively moderate number of large bundles (major tracts), axons must also follow more complex trajectory patterns to reach their gray matter targets.

Another consequence of a highly dense network of white matter connections is that analyzing network organization in terms of mere existence or absence of a connection (that is, a binary analysis) becomes inherently limited. Instead, other network descriptions

may be more informative, such as intra-area organizations, or quantitative information on connection 'strength', such as the number, density and caliber of axons connecting two regions. In addition, tracer studies have revealed quite complex spatial or topographic organization 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 (for example, biases toward sulci or gyri). Summarizing such rich connectivity patterns in terms of an area-to-area matrix, although a useful conceptualization of brain networks, may hide important and functionally relevant organization.

Examples of connection patterns that may pose challenges to *in vivo* imaging methods (see below) are shown in **Figure 1a**,**b**. Ipsilateral and contralateral long-range connections converge in the principal sulcus in the macaque monkey[9] (**Fig. 1a**). Their terminal fields can display striking interdigitation, a pattern that would need imaging at relatively high spatial resolution to be captured. An example of laminar organization of cortico-cortical connections is shown in **Figure 1b**. Such a pattern would require the ability from an imaging modality to separate connections to and 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] (**Fig. 1c**). 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 to be the gold standard in measuring connections, as it has two important strengths. First, there is a low false positive rate. Detection of the injected compound away from the injection site is very strong evidence for a connection. Second, the spatial resolution allows the discovery of the detailed organizations (**Fig. 1**). Tracer studies, however, also have several caveats, of which two are particularly relevant in this case. First, assignment of the injection site to a particular brain area is not necessarily known, particularly when no clear cytoarchitechnonic 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 to build a normative area-to area connectome. Second, it is difficult to quantify 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 it is therefore only a proxy for connection strength. Anterograde tracing can reveal the extent of terminal fields, but using these techniques to quantify connections (for example, number of terminal buttons) is both technically difficult and vastly labor intensive.

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

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. In addition, these tools cannot easily be used in conjunction with functional or behavioral measurements. *In vivo* imaging methods, such as diffusion and functional MRI (fMRI), 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. 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.

Diffusion MRI and 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, it is 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 organized, such as in white matter, water diffusion is anisotropic, in that diffusion is less hindered parallel than perpendicular to axons. Thus, by measuring the orientational dependence of water diffusion, we can estimate axonal orientations *in vivo* (**Fig. 2a**). Tractography algorithms use local information on orientation to infer long-range connections (**Fig. 2b**) and allow us to perform *in vivo* 'virtual dissection' of white matter bundles[15]. By repeating tractography

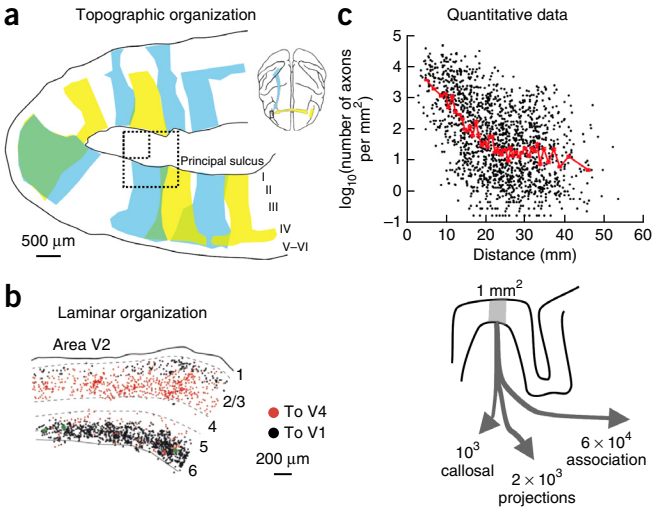

Figure 1 Examples of complex white-matter organization in monkey brains. (**a**) Topographic organization of afferent ipsilateral and contralateral connections to the principal sulcus in the macaque brain showing columnar interdigitation. Typical 1-mm and 0.5-mm isotropic voxels are shown as dashed boxes, indicating the resolution needed to reveal such macro-scale organization (modified with permission from ref. 9). (**b**) Laminar pattern of efferent connections from visual area V2. Cells connecting to V1 sit primarily in superficial and deep layers, whereas those connecting to V4 originate from layer 2/3. No labeled cells are found in layer 4 (modified with permission from ref. 115). (**c**) Top, estimate of the number of efferent ipsilateral association axons from 1 mm^2 of cortex as a function of the distance between source and target regions. Bottom, estimate of the number of axons from 1 mm^2 of cortex forming different categories of fiber tracts. Estimates are based on ref. 1 and data from http://core-nets.org.

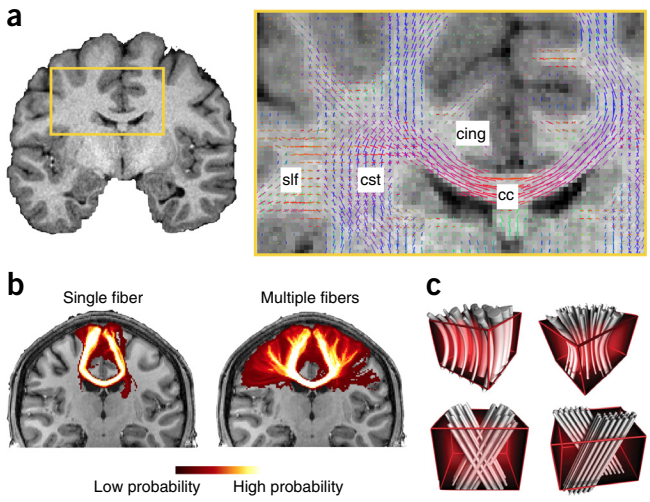

Figure 2 Diffusion MRI and tractography. (**a**) A coronal section through a human brain (left) and estimated fiber orientations from diffusion MRI data (right). Voxel-wise fibers are color-coded according to their orientation (red = left-right, green = anterior-posterior, blue = ventral-dorsal). Major fiber bundles can be visualized on the orientation maps (cing = cingulum bundle, cc = corpus callosum, cst = cortico-spinal tract, slf = superior longitudinal fasciculus). Note the many voxels with multiple orientation estimates (crossing fibers) 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 are from the Human Connectome Project[65,116]. (**b**) Probabilistic tractography of the corpus callosum pathways consists of 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 fiber orientation in a voxel is not always representative of the underlying anatomy. Crossings of fiber bundles are very common in white matter. The figure shows probability maps arising from the body of the corpus callosum when modeling multiple versus single fiber orientations. Ignoring fiber 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 modeling voxel-wise fiber orientations. Four different putative voxel-wise patterns of axonal organization can give rise to the same diffusion scatter pattern when averaged over a voxel. Top left, bending fibers; top right, 'kissing' fibers; bottom left, inter-digitated fibers; bottom right, 'touching' fibers. Simple crossing fiber modeling cannot distinguish these cases, which may lead to false positive and false negative connections. Figure reproduced with permission from ref. 117.

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 their early days, considering the many technical and conceptual challenges that remain to be addressed to improve their interpretability. Many of the technical limitations are well known in the community of *in vivo* connectomics[16–18], and addressing them is the object of intense research (for example, see refs. 19–22 for new methods or refs. 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 (electron) microscopy, where individual axons can be visualized 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 ref. 25 for a more complex view). However, considering that a white matter voxel may contain hundreds of thousands of axons, unless all of these axons are well aligned, the mapping from diffusion to axonal orientations is often ill-posed (all these patterns are likely to give rise to the same MRI measurement; **Fig. 2c**). 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 previously[27]: white-matter trajectories estimated using tractography were compared to a macaque atlas[28]. It was found that the sensitivity and 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 (**Fig. 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 a result of the resolution of diffusion MRI data and of superficial white-matter bundles (running parallel to the cortex) impeding the detection of fibers 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 subcortical terminations, both because these connections tend to be organized into coherent bundles and because the subcortical targets have simpler topologies than the cortical convolutions.

Limitations resulting from 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 modeling 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, caliber, 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 modeling and/or more advanced diffusion MRI sequences[32–35].

Pending these advances in quantification of white-matter connections, it is possible to calculate semiquantitative 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 (normalized) variance of the diffusion coefficient along all directions in three dimensions under the assumption of anisotropic

Gaussian diffusion[37]. A number of experiments in animal models have shown how FA relates to myelination, membrane permeability and fiber density in white matter[38–42]. Although FA may reflect fiber integrity, it can also 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 (for example, see ref. 38), making this another important, but complex, issue to consider in quantitative tractography analyses.

In addition, 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 fiber orientations to the diffusion data and building pathways through these orientation fields. Several true anatomical factors (for example, axon density) can influence these probabilities, but other confounding factors can also contribute significantly. These include for instance the distance traveled 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 millimeter-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.

fMRI: 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. Thus, 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 fMRI (R-fMRI) connectivity was first demonstrated in ref. 47, where it was shown to detect strong functional connectivity in 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 fMRI (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 coactivation maps[48]. The motivation for using coactivation 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 coactivation and R-fMRI correlations[50]. Using independent component analysis, the study found remarkable similarities in several task coactivation networks and resting-state networks.

Functional connectivity has been reviewed extensively elsewhere (for example, see refs. 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.

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

Second, 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 in which 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 to explain earlier observations of intact bilateral functional connectivity in patients with agenesis of the corpus callosum[58].

Comparing connectivity inferences with fMRI 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, fMRI 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 (**Fig. 1b**). With increasing spatial resolution offered by ultra high–field (7 T) 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].

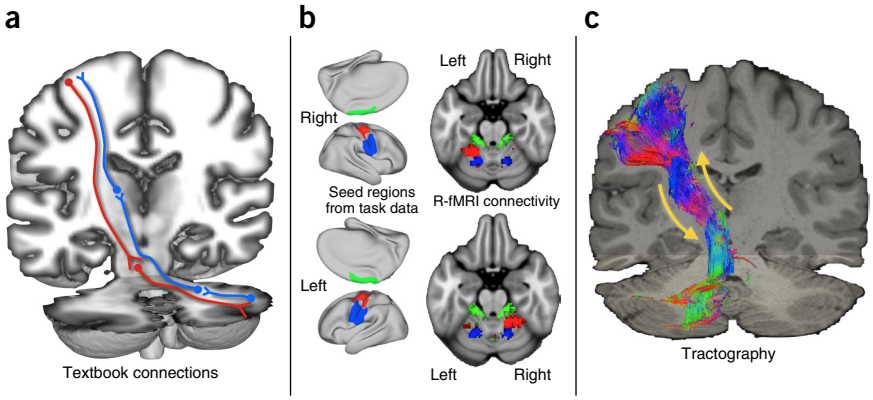

Figure 3 Textbook and estimated cortico-cerebellar connections using functional and diffusion MRI. (**a**) Multi-synaptic efferent (blue) and afferent (red) cerebellar connections decussate at the level of the pons. (**b**) Resting-state functional connectivity of motor cortical regions to cerebellum (foot, hand and face area as green, red and blue, respectively). The somatotopic organization is evident, but notice that, except for the hand area, homolog 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 as a result of the smoothness constraints used in tracking algorithms.

In contrast, diffusion MRI tractography cannot reveal laminar-specific connections regardless of the spatial resolution.

Although 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 (**Fig. 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[60,61], functional connectivity of the motor cortex and cerebellum correctly recovers the somatotopic organization of these connections (**Fig. 3b**). However, only the hand representation 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 both indirect and 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 (**Fig. 3c**). This is a result of both the complex geometry of white matter at the decussation and of 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 fibers in white matter can accurately represent the local orientations associated with these connections, but tractography algorithms use rules that do not favor sharp turns. At a crossing fiber voxel, tractography algorithms select the fiber orientation most aligned with the direction of the incoming pathway. Although 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 while simultaneously 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-cortical 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 in which 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 (**Fig. 4d–f** and see ref. 63).

To summarize, functional connectivity can be used to infer structural long-range connections. Similar to 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, R-fMRI, and T-fMRI data from a large number of healthy adults in the Human Connectome Project[65] provides an excellent substrate for exploring these issues in detail.

The need for white matter organization. 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 organization of white-matter bundles is proposed, and we postulate that connections that do not fulfill 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 6×10^9 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 millimeter of cortex on average sends and receives 6×10^4 axons going to or coming from other cortical regions of the same hemisphere. These 60,000 axons are distributed into short-, middle- and long-range projections. Recent quantitative tracer data in the macaque revealed that the number of cortico-cortical axons decreases exponentially with the distance between the source and target area[10]. For instance, although 40% of these axons project to within 10 mm, fewer than 1% travel a distance of 35 mm or more (the distance between, for example, primary visual cortex and anterior

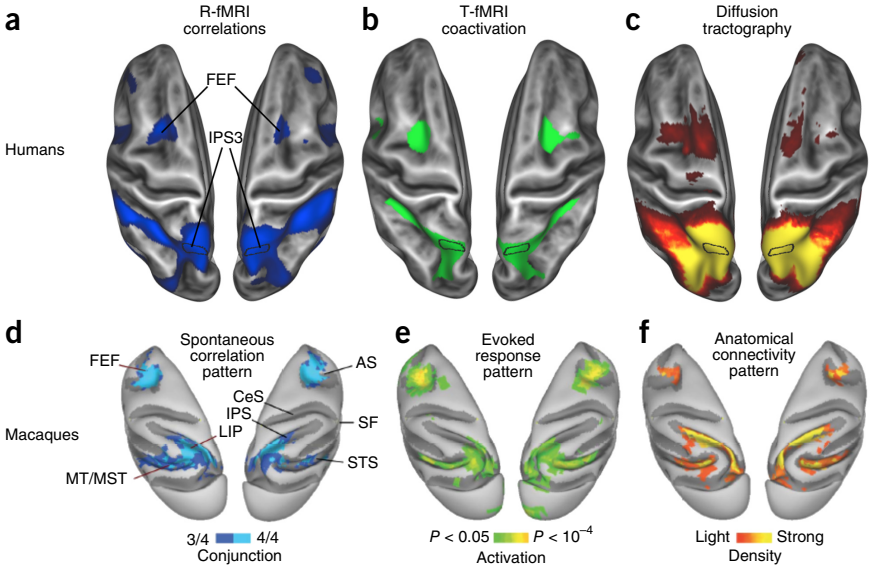

Figure 4 Agreement between functional and structural connectivity in measuring connections in human and macaques. (**a**–**c**) Connectivity maps for area IPS3 obtained from data acquired for the Human Connectome Project[65] (average connectivity of 40 unrelated subjects). (**a**) Functional connectivity using correlations of R-fMRI time series. (**b**) Functional connectivity using correlations of activations across multiple tasks (7 tasks and 42 contrasts). (**c**) Structural connectivity using diffusion MRI and probabilistic tractography. (**d**) Map of voxels exhibiting BOLD correlations in spontaneous activity amongst at least three of four regions of the oculomotor system in the anesthetized 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 labeled by retrograde tracer injections into LIP (average of three monkeys). Images in **d**–**f** are adapted with permission from ref. 63.

temporal lobe in macaques). Considering that a white-matter voxel at high spatial resolution may contain 3×10^5 axons, this means that the contribution of axons projecting from a distant square millimeter of cortex may be about 0.2% of a white matter voxel near the target, that is, 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. In addition, some of the long association pathways may be substantially stronger than others and the contribution of a square millimeter 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, 1–2,000 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], that is, if we do not summarize the connections over large regions spanning many square millimeters.

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 organization. Given the spatial scale of *in vivo* techniques relative to that of axons, white-matter axons are bound to intermix. 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 and termination. This may be possible if the intermixing is non-random, but spatially organized, such that the axonal inputs and outputs for neighboring cortical regions tend to occupy neighboring pieces of white matter. If such spatial organization is not present or not measurable, then a different postulate of (statistical) white-matter organization 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 in 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 organization, to the extent that it is respected, may alleviate the intermixing issues resulting from scale differences between axons and voxels. However, the degree to which white matter is organized along such lines is not known. One can hope that spatial organization 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 previously[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, that is, generating accurate large-scale structural connectomes.

Evidence for organization. Although a complete picture of white-matter organization, particularly for the human brain, is not currently available, evidence in favor of this hypothesis of spatial organization 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 organization. For instance, projections from the macaque orbitofrontal cortex appear to follow a spatial organization dictated by the medial-lateral position of their origin[69]. The same organization 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 organization. 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 organization 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 behavior. If all tractography results are artifactual, 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.

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 behavior 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], whereas latest technology makes neonatal and fetal 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). Here we give some representative examples of these applications, focusing on demonstrations that provide indirect evidence of the accuracy of *in vivo* methods.

Relating structure to function and behavior. 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 organization that is necessary for long-range tracking to succeed.

Tractography has been used to identify locations in the thalamus that preferentially connect to particular cortical regions[76]. When these 'connectional localizers' are compared to 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 (SEPs) recorded using electrodes covering an extended area of the thalamus revealed a restricted area of thalamus in which 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 localization is deep brain stimulation of subcortical 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 gray matter region constitute a signature of its functional role[2], it should be possible to identify functional boundaries by searching for cortical locations that exhibit sharp transitions in connections[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]. However, the extent to which these parcellation techniques are affected by issues such as the gyral bias remains unclear. Determining the boundary between cortical regions using tractography should ideally be augmented with evidence from other modalities, for example, using the aforementioned multimodal Human Connectome Project data sets.

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 favorable organization.

Finally, there is growing evidence that (semi-)quantitative measurements from tractography can predict regional functional and cross-subject behavioral variation. A good example is provided by a network of cortical and subcortical regions known to be involved

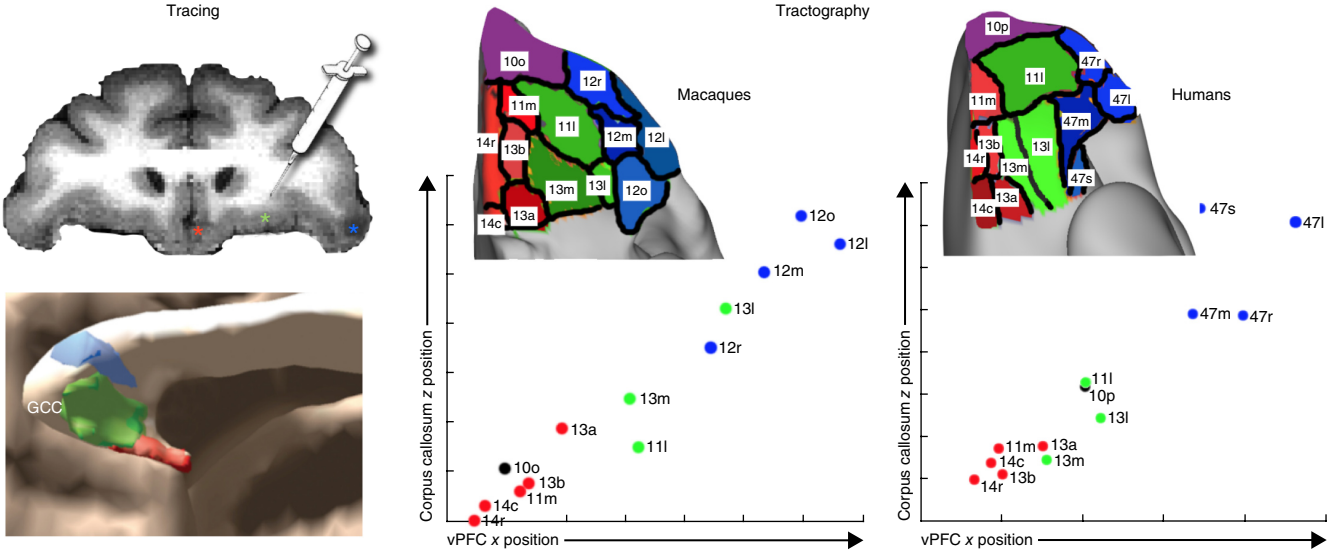

Figure 5 Testing generic organization principles using tractography. Injection of tracers into three locations of the macaque vPFC reveals that the medial-lateral position of the injection sites in the vPFC dictates the relative position of the corresponding pathways in the genu of the corpus callosum (GCC). Tractography is then utilized 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 ref. 105 for macaques and ref. 104 for humans. The regions are colored in red, green and blue according to their approximate medial-dorsal positions for ease of visualization. Figure adapted with permission from refs. 26,69.

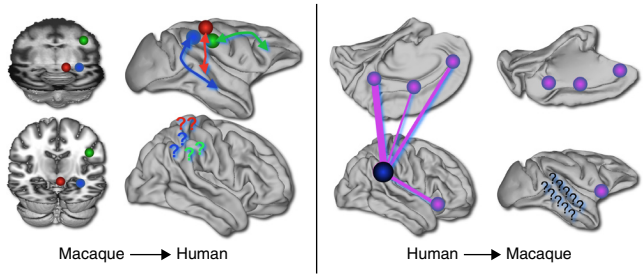

Figure 6 Finding homolog areas across species. Left, three areas surrounding the macaque intra-parietal sulcus have been found through tracer studies to have distinct connection patterns to the superior colliculus (red), parahippocampal gyrus (blue) and ventral premotor cortex (green), respectively. 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]. Right, activity in the TPJ is associated with 'theory of mind', the human ability to infer the 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 homolog area can be found by searching for areas in the macaque cortex with the same connectivity profile[108].

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 behaviors[93].

The observed correlation between structural connections and behavior 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 (VS) vary between subjects and have substantial consequences on ventral striatal functional responses. The BOLD signal in 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 VS 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.

Learning and augmenting organizational principles. In animal models, it is possible to combine diffusion MRI tractography with tract tracing 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.

Previously[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] generalize to the entire vPFC. Injections of tracers into three locations of vPFC suggest a pattern of organization for these pathways: the medial-lateral position of the injection sites in the vPFC dictates 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 organization not only generalizes across the vPFC, but also across species. Similarly, R-fMRI data have been recently used to map the organization of connections between vPFC 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 analyze spatial organization.

Comparative anatomy across species. The vast amount of electrophysiological and neuroanatomical data from nonhuman primate studies provides a framework for interpreting human data. But comparative studies of connectional 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 homolog areas across species. This was traditionally achieved using combinations of macroscopic morphological landmarks and cytoarchitectonic markers. For instance, 13 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 homolog 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 subregions surrounding the intra-parietal sulcus with well-documented connection patterns from anatomical tracer studies. These three parietal regions can be distinguished by a subset of their anatomical connections (see **Fig. 6**). In particular, three well-separated targets can be used as a signature for the parietal areas, providing candidate homolog 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 uniquely determine TPJ's connectivity and 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].

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 as a result of 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 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 behavioral measurements with connectivity is a real asset that tracer techniques cannot replace.

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