Recent technological developments have renewed the interest in large-scale neural circuit reconstruction. To resolve the structure of entire circuits, thousands of neurons must be reconstructed and their synapses identified. Reconstruction techniques at the light microscopic level are capable of following sparsely labeled neurites over long distances, but fail with densely labeled neuropil. Electron microscopy provides the resolution required to resolve densely stained neuropil, but is challenged when data for volumes large enough to contain complete circuits need to be collected. Both photon-based and electron-based imaging methods will ultimately need highly automated data analysis, because the manual tracing of most networks of interest would require hundreds to tens of thousands of years in human labor.

Resolution requirements
The reconstruction of a complete neural circuit (which can be described by the connection strengths between all possible pairs of neurons) requires for each pair of neurons that first, the somata are identified; second, both axons and dendrites are followed; and third, synapses are detected and their strengths estimated (Figure 1b). If the neurons carry specific labels (e.g. distinct colors), continuity of neurites (but not necessarily their synaptic connectivity, see below) can be inferred from the staining color alone (Figure 1b), allowing for an imaging resolution coarser than the minimum neurite diameter. If the neurons are not specifically labeled, however, then each neurite must be individually resolved. The same applies to synapse identification. If synapses are not specifically stained, characteristic features (such as vesicles, postsynaptic densities, or synapse geometry) have to be spatially resolved. Synapses between a pair of neurons can be inferred directly if the labeling depends on the existence of a synapse (as is the case for trans-synaptic virus propagation, see below).

In special cases, such as when axons run in parallel bundles, the imaging resolution can be coarser along the bundle direction than perpendicular to it (Figure 1c, top panel). If, however, the neurite direction can be along any axis in space, as is typical for neuropil, a resolution substantially finer than the minimum neurite diameter is needed along all spatial directions (Figure 1c, middle panel). The imaging resolution can be much lower if specific or sparse staining is employed such that, within a resolution element (voxel) no two neurites from different neurons have the same label (color). The required resolution then depends on the sparseness of the staining and on the number of different labels (Figure 1c, bottom panel). Given a sufficient number of colors even conventional light microscopy (LM) can provide sufficient resolution to follow each neurite even when every cell is labeled. The currently available staining techniques, imaging methods, and reconstruction tools are summarized in Tables 1–3.

Brainbow
The almost complete intracellular staining of a small fraction of all neurons allows neurites to be followed that are too small to be resolved from their neighbors at the light microscopic level. This has been the basis for most cellular neuroanatomy since Ramón y Cajal [8]. The Golgi method [9] and other methods such as biocytin–HRP–DAB staining [10] are monochrome and require that resolution volumes are not shared by different processes, limiting reconstruction to rather sparsely labeled...
tissue. If instead we have a number of independent colors one can simply increase the labeling density by that number (independent colors, in analogy with primary colors, can be separated by spectral filtering even when voxels contain more than one color). The situation becomes more complicated if we have colors that are merely distinguishable, that is, colors that are only distinct as long as a voxel contains just a single color. A small number of independent colors can be used to create a large number of distinguishable colors. One example is color television (three independent colors), another is the 'brainbow' technology [11,12] where randomly varied mixtures of up to four spectrally different fluorescent proteins (GFP, YFP, CFP, and RFP) are used to generate about 100 distinguishable colors. In analogy to a cable harness containing color-coded wires, this allows the identification of neural processes at both origin and the termination of a neural projection without actually tracing the wires along the harness. For this it is crucial that the color is constant throughout the entire cell. Ideally, each neuron would express a unique color. For the Thy-1 promoter, which is used for the current implementation of brainbow, the fraction of neurons that show expression can be quite variable [13]. Because brainbow colors are not independent a neurite can only be uniquely identified when its diameter exceeds the size of the resolution volume. This is not the case for many nonmyelinated axons [14] and many spines [15] at the resolution of conventional LM. Superresolution LM [16–18] may improve the situation. Synapses can in some — but not all — cases be inferred from the morphology of neurite contacts at the LM level. Synapse-specific immuno-labeling or XFP-labeling might improve the fidelity of synapse identification.

Trans-synaptic tracing
A potentially more reliable method to establish synaptic connectivity is the use of trans-synaptic viral infection [19–21], which has recently been improved so as to limit infection to a single retrograde trans-synaptic hop [22,23].
using a deletion-mutant rabies virus [24] (Table 1). This method works by first transfecting a very sparse set of neurons (using, for example, a gene gun or in vivo electroporation) with the DNA encoding a specific receptor for the modified rabies virus, the glycoprotein required for trans-synaptic spread (which is missing in the deletion-mutant virus), and DsRed2 as a label for transfected neurons. The virus can then only infect neurons that express the specific receptor. It spreads trans-synaptically to a large fraction of presynaptic neurons [20], where the virus genome replicates and expresses EGFP. Lacking the capsule protein, it cannot, however, spread any further. The mechanism of spreading identifies the synapse (if a neuron is green it is presynaptic to one of the red neurons) and allows a very efficient sampling of pairwise connectivity for a given postsynaptic neuron. For the technique to become useful for dense reconstruction, it needs to be combined with a method to increase the color space, such as brainbow, and/or with superresolution fluorescence imaging.

### Serial section transmission electron microscopy (SSTEM)

The high lateral (xy, in-section) resolution of the transmission electron microscope (TEM) in combination with serial sectioning has enabled the reconstruction of neural circuits. Limitations include the painstaking process of manually cutting thousands of sections, and the need for

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Table 1

<table>
<thead>
<tr>
<th>Method</th>
<th>Applicable imaging methods</th>
<th>Sampling (% of neurons in a given volume)</th>
<th>Order of connectivity resolvable</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sparse intracellular staining (e.g. biocytin–HRP–DAB)</td>
<td>LM, TEM, SEM</td>
<td>0.001–1% (1–10 of 1000–10000 neurons)</td>
<td>Pairs</td>
<td>[51]</td>
</tr>
<tr>
<td>Brainbow</td>
<td>LM</td>
<td>&gt;75% (motor axons, mossy fibers)</td>
<td>∞ for the stained neurons</td>
<td>[12**,13]</td>
</tr>
<tr>
<td>Trans-synaptic tracing</td>
<td>LM</td>
<td>1 postsynaptic neuron, high fraction of presynaptic neurons</td>
<td>High fraction of pairs for a given postsynaptic neuron</td>
<td>[23]</td>
</tr>
<tr>
<td>Conventional en-bloc and postsectioning EM stains (osmium, uranyl acetate, and lead citrate)</td>
<td>TEM, SEM</td>
<td>100%</td>
<td>∞</td>
<td>[52] [36]</td>
</tr>
<tr>
<td>Surface HRP staining</td>
<td>TEM, SEM</td>
<td>100%</td>
<td>∞</td>
<td>[48], KL Briggman, W Denk, unpublished data</td>
</tr>
</tbody>
</table>

Images from [12**,23,51,52]; KL Briggman, W Denk, unpublished data.

a The neuron sampling ratio for the brainbow technique applies to the current implementation using the Thy1 promoter [12**,13].

b Assuming that synapses can be identified, discussed in the text.
An even more fundamental problem is that the resolution in the \( z \) direction (perpendicular to the sectioning plane), which is determined by the section thickness (\( \geq 45 \text{ nm} \)), is coarser than that required to follow thin in-plane processes reliably. For thick (>200 nm) neurites or oriented axon tracts a slicing thickness of 45 nm is sufficient. Most published SSTEM studies comprise, at most, a few

### Table 2

<table>
<thead>
<tr>
<th>Imaging method</th>
<th>Cutting method</th>
<th>Voxel size [nm(^3)]</th>
<th>Number of sequential slices</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM</td>
<td>Optical sectioning</td>
<td>((200-500)^3)</td>
<td>For example, 100-300 ( @ ) 500-1000 nm for 50-150 ( \mu \text{m} ) of fixed tissue [53]</td>
</tr>
<tr>
<td>TEM</td>
<td>Manual</td>
<td>(\sim 4 \times 4 \times 43-100)</td>
<td>&gt;8000 ( @ ) 50 nm [27]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1200 ( @ ) 100 nm [54]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1073 ( @ ) 50 nm [28]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>214 ( @ 43 \text{ nm} ) [55]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>319 ( @ 90 \text{ nm} ) [56]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>270 ( @ 70 \text{ nm} ) [45]</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>200-300 ( @ 80 \text{ nm} ) [57]</td>
</tr>
<tr>
<td>SEM</td>
<td>ATLUM</td>
<td>(\sim 4 \times 4 \times 25-50)</td>
<td>600 ( @ 25 \text{ nm} )</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>&gt;1000 ( @ 35-40 \text{ nm} ) (JW Lichtman, personal communication)</td>
</tr>
<tr>
<td></td>
<td>FIB-SBFSEM</td>
<td>(\sim 4 \times 4 \times 15-40)</td>
<td>120 ( @ 40 \text{ nm} ) [33**]</td>
</tr>
<tr>
<td></td>
<td>Diamond-knife SBFSEM</td>
<td>(20 \times 20 \times 25)</td>
<td>2000 ( @ 25 \text{ nm} ) (KL Briggsman, W Denk, personal communication)</td>
</tr>
</tbody>
</table>

### Table 3

<table>
<thead>
<tr>
<th>Method</th>
<th>Staining-imaging combination</th>
<th>Speed [hours per millimeter neurite length]</th>
<th>Reconstructable total neurite path length (2000 hour human work load(^a))</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Manual volume labeling</td>
<td>Conventional — TEM</td>
<td>200–400 hours/mm(^b) 5–10 mm</td>
<td></td>
<td>[43,58]</td>
</tr>
<tr>
<td></td>
<td>Single neuron staining — LM</td>
<td>0.25–1 hours/mm(^c) 2000–8000 mm</td>
<td></td>
<td>[51,59]</td>
</tr>
</tbody>
</table>
hundred sections with section thicknesses between 45 and 100 nm (Table 3). A few studies have used substantially larger numbers of sections. For the reconstruction of the C. elegans wiring diagram, [27] and for the neural plexus of the horseshoe crab eye [28] ~5000 and >1000 serial sections were cut at 50-nm thickness. SSTEM analysis of intracellularly stained cells, in combination with prior LM, has also been used to reliably

Table 3 (Continued)

<table>
<thead>
<tr>
<th>Method</th>
<th>Staining–imaging combination</th>
<th>Speed [hours per millimeter neurite length]</th>
<th>Reconstructable total neurite path length (2000 hour human work load)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brainbow — LM</td>
<td>8–12 hours/mm^1</td>
<td>170-250 mm</td>
<td></td>
<td>[11,12**]</td>
</tr>
<tr>
<td>Extracellular — SBFSEM</td>
<td>200–400 hours/mm</td>
<td>5–10 mm</td>
<td>M Helmstaedter, KL Briggman, W Denk, unpublished observations</td>
<td></td>
</tr>
<tr>
<td>(Semi)automated volume labeling</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brainbow — LM</td>
<td>2 hours/mm</td>
<td>1000 mm</td>
<td></td>
<td>[40–42,60]</td>
</tr>
<tr>
<td>Conventional — SSTEM</td>
<td>40 hours/mm^d</td>
<td>50 mm</td>
<td></td>
<td>[61]</td>
</tr>
<tr>
<td>Conventional — SBFSEM</td>
<td>5.5 hours/mm</td>
<td>400 mm</td>
<td></td>
<td>[62,63]</td>
</tr>
<tr>
<td>Skeletonization</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Extracellular — SBFSEM</td>
<td>10–20 hours/mm</td>
<td>100–200 mm</td>
<td>M Helmstaedter, KL Briggman, W Denk, unpublished observations</td>
<td></td>
</tr>
</tbody>
</table>


a For comparison: 1 cortical column contains approximately 240 000–400 000 mm neurite path length (assuming 12 000 neurons with 20 mm path length, or using volume-density estimates summarized in [1]).
b Assuming a neurite density of 4.5 km/mm^2 of cortex [1] and a tracing speed of one to two hours per µm^3 [43]. Quantification of all volumes and identification of individual synaptic structure can take considerably longer (K Harris, personal communication).
c D Feldmeyer, personal communication.
d Tracing speed: 0.2 hour per µm^3 [61].
e For data from the Calliphora vicina outer chiasm: 15 s per image with 15 neurites, slice thickness 50 nm [62].
f JW Lichtman, personal communication.
identify synaptic structures [29–31], a task for which SSTEM is well suited because the high xy-resolution allows the recognition of synaptic vesicles and postsynaptic densities. Tight junctions and gap junctions can only be identified in certain orientations, however.

**Automated section collection**
Some of the limitations of SSTEM can be overcome by an automatic section-collection process. Sections can be cut with a rotary ultra-microtome and collected continuously onto a carbon-coated polymer tape with the Automated Tape-collection Lathe Ultra Microtome (ATLUM) [32**]. The tape precludes viewing in the TEM but modern SEMs with field-emission emitters provide comparable resolution and contrast in back-scattered electron mode [32*,33**,34*,35]; (Briggman, Denk, unpublished data). Automated collection reduces the likelihood of lost or damaged sections and appears to reduce section distortion during imaging, presumably because of the supporting substrate (JW Lichtman, personal communication). ATLUM also, for reasons poorly understood so far, allows a reduction of the section thickness to about 25 nm, maybe even less (JW Lichtmann, B Kasthuri, personal communication). Sections can be stained using standard contrast-staining protocols [36] and remain available for repeated imaging, potentially even for postembedding labeling with antibodies [35].

**Block-face imaging**
Section distortion is one of the main problems preventing the assembly of high-quality 3D EM data. Distortion (and the need to collect sections) can be avoided if each slice is imaged before sectioning. This requires imaging the block face of an embedded sample with sufficient resolution and depth discrimination, which is possible with focused ion-beam (FIB) milling [39]. This approach is also less sensitive to electron-beam-induced damage to the resin, which affects cutting in diamond-knife SBFSEM [34*] (Briggman, Denk, unpublished data). The insensitivity to beam damage allows the use of higher electron doses and hence higher resolution and a better signal-to-noise ratio. Knott et al. [33**] thus were able to record images at a lateral resolution of 4 nm, with good visibility of synaptic vesicles and densities. A crucial issue is the uniformity of the section thickness. Often only the microtome advance or the mitochondria thickness are reported, both of which are not sensitive to variations of the section thickness between subsequent cuts. Cutting problems are often apparent in resliced images along the z-direction.

The size of the volume that can be imaged depends, of course, on the number of sections that can be taken in unbroken sequence. It also depends on the block-face area that can be imaged. With ATLUM and diamond-knife SBFSEM one limitation is the width of the diamond knife, which can, however, be several millimeters. In FIB-based SBFSEM the block-face area is currently limited by what can be milled off in a reasonable time and without artifacts, such as surface ripple [33**]. For large block faces the imaged area needs to be divided into multiple tiles because of the limited field of view (because of scan-generator restrictions and off-axis electron-optical aberrations) of the SEM at high resolution. The acquisition of well-aligned 3D image stacks with sufficient resolution to trace most neurites has been demonstrated with diamond-knife SBFSEM (Briggman, Denk, unpublished observations) and appears feasible with ATLUM and FIB-SBFSEM.

**Reconstruction**
The ultimate aim of imaging the structure of neural circuits is to construct neural connectivity matrices (Figure 1a). To achieve this, neurites must be traced through 3D data and synapses between neurites need to be detected and their strength estimated (Tables 1 and 2). A major challenge is following neurites through neuropil with high reliability (Figure 1b). That the human visual system can perform this task demonstrates that the raw data contains sufficient information. Manual tracing can yield valuable insight, but it is time consuming. At the LM level, it takes 0.25–1 hour per mm neurite path length (D Feldmeyer, personal communication). Thus it takes roughly 30 hours to completely trace all neurites belonging to a single intracellulary stained spiny stellate neuron in L4 of rat barrel cortex. Some semiautomated techniques mimic the manual labeling procedure by inferring new contours from already drawn contours in adjacent slices [40–42].

Manual reconstructions from SSTEM image stacks are even slower (one to two hours per μm² of gray matter, i.e., 200–400 hours per mm neurite path length,
assuming a neurite density of 4.5 km/mm$^3$ [1]). Without alternative, manual reconstruction has been used in a number of heroic reconstruction efforts, such as, over a period of 15 years (JG White, personal communication, [26]) virtually the entire nervous system of C. elegans [27] (see also [44]). SSTEM-based manual reconstructions of small volumes, $\sim(10–20\ \mu m)^3$ of neural tissue are mostly used to explore details of synaptic structure or of neuron–glia interaction (e.g. [14,45,46]) sometimes in correlation with LM (e.g. [29,31]). For the analysis of complete neuronal wiring diagrams, manual reconstruction of SSTEM images appears to be prohibitively expensive in terms of human labor (24 000–80 000 work years for a cortical column, cf. Table 3).

High contrast and the minimization of fixation artifacts are crucial even for entire manual reconstructions. Staining strategies designed to emphasize the cell surface can further aid manual reconstruction and are likely to be necessary for automatic techniques. Surface staining makes it easy to follow the unstained cell lumen through the volume and can be achieved by the infiltration of horseradish peroxidase (HRP) into the living tissue followed by fixation and a standard DAB staining and OsO$_4$ into the living tissue followed by fixation and a standard DAB staining and OsO$_4$ into the living tissue (see also [44]). SSTEM-based manual reconstructions of small volumes, $\sim(10–20\ \mu m)^3$ of neural tissue are mostly used to explore details of synaptic structure or of neuron–glia interaction (e.g. [14,45,46]) sometimes in correlation with LM (e.g. [29,31]). For the analysis of complete neuronal wiring diagrams, manual reconstruction of SSTEM images appears to be prohibitively expensive in terms of human labor (24 000–80 000 work years for a cortical column, cf. Table 3).

Well-aligned 3D data from cell surface-stained material are promising for computer-based segmentation methods not only because of the high contrast but also because of the availability of an undistorted surround in all three dimensions. This makes this problem amenable to machine-learning-based analysis such as memory-based pattern matching (Helmstaedter, Briggman, Denk, unpublished data), convolutional neural network classifiers [49], or hierarchical random-forest classifiers [50]. Crucial for any machine-learning method is the availability of high-quality training data (Helmstaedter, Briggman, Denk, unpublished data) and of test data that can evaluate the reliability of automatically generated segmentations over longer distances. The training data are best generated using careful tracing of a small volume. To test its performance, it is crucial to know whether an automatic segmentation procedure correctly predicts whether two distant points belong to the same neurite or not. This can be tested using manually generated skeletons (tree-like structures comprising lines running roughly along the centers of neurites). Given appropriate software tools (Table 3), skeletons can be generated in SBFSEM data at a speed of approx. 15 hours per mm neurite path length (Helmstaedter, Briggman, Denk, unpublished data), which is more than 10 times faster than volume labeling. The figure-of-merit for any algorithm will not be so much the speed at which it runs on the computer but, rather, by which factor it reduces the total human workload, including any proofreading and error-correction steps. To make the complete reconstruction of, for example, a cortical column feasible (1 work year) the workload needs to be reduced several thousand times compared to that needed to manually trace skeletons (1200–4000 work years for a cortical column, Table 3). To us the increase of the analysis speed seems much more important than speeding up acquisition. Even with SBFSEM, where voxel rates are currently limited to well below a megavoxel/s, mostly by the need to use low beam energies, it should be possible to take data for an entire cortical column ($\sim300\ \mu m \times 300\ \mu m \times 1000\ \mu m$; nine tera voxels) in fewer than two years. Sufficiently fast analysis will only be possible if the segmentation algorithm is in itself reliable enough or else reliably pinpoints locations where human interaction is needed. The necessary overall reliability depends on the error rate that one is prepared to accept in the connection matrix, which in turn depends on the hypotheses that need to be tested. For a reliability of the cortical-column connectivity matrix of, say, 99% (i.e. accepting that 1% of all neurons contain a tracing error), we need a tracing error rate of fewer than 1 error per 2 m of neurite path length (assuming a neurite path length of $\sim20\ \mu m$ per cortical neuron).

Summary
We have reviewed the techniques currently being developed for imaging the connectivity of neural circuits. For the reconstruction of small neural circuits (up to 100 $\mu m$ on a side) at high resolution, the FIB method looks promising. For the reconstruction of long-range projections, LM-based techniques such as Brainbow or EM-based techniques used on a coarser scale (cutting of $>200\ \mu m$-thick slices) are well suited. For the reconstruction of medium-sized local circuits in their entirety (e.g. a $300\ \mu m \times 300\ \mu m \times 100\ \mu m$ volume of retina, or a cortical column) mechanically cutting techniques such as SBFSEM or ATLUM may be the most suitable. Essential but still unsolved is the automated reconstruction of the data.

Acknowledgements
We thank DB Chklovskii, T Euler, KM Harris, V Iain, JW Lichtman, HS Seung, and S Turaga for helpful discussions and HS Seung for comments on the manuscript. We thank A Bors, E Callaway, D Feldmeyer, KM Harris, JW Lichtman, Y Mishchenko, B Sakkmann, and I Wickersham for figure contributions.

References and recommended reading
Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

New technologies


34. Heymann JA, Hayles M, Gestmann I, Giannuzzi LA, Lich B: Focused ion-beam milling for serial block-face scanning electron microscopy and may be the technique of choice for the complete reconstruction of small volumes (less than 100 µm on a side) at high resolution.


38. Leighton SB: SEM images of block faces, cut by a miniature diamond knife microtome mounted inside a SEM. This technique is promising for obtaining data on neuronal connectivity matrices from intermediate size volumes of dense neuropil (volumes of several hundred micrometers on a side).


41. Heymann JA, Hayles M, Gestmann I, Giannuzzi LA, Lich B: Focused ion-beam milling for serial block-face scanning electron microscopy using a diamond-knife microtome mounted inside a SEM. This technique is promising for obtaining data on neuronal connectivity matrices from intermediate size volumes of dense neuropil (volumes of several hundred micrometers on a side).


