provide an anatomical road map for thinking about information processing. Additionally, if obtaining such data were straightforward, projectomes of normal adult brains could be readily compared to each other to check for variations in brain connectivity or compared to those of neurological mutants, very young or old brains. All of these comparisons could yield new and interesting insights. Given that such a map is of the forest rather than the single trees or leaves, the requirements for resolution are not as stringent as many new techniques provide. What is needed though, is a means of imaging a large volume, preferably an entire nervous system as a single entity.

The prospects for creating projectomes have improved greatly with the addition of the two new imaging techniques described in this issue. The first, by Verveer *et al.*[6], has similarities to a previously described technique optical projection tomography (OPT[7]) and improves upon their earlier description of single-plane illumination (SPIM[8]). Unlike traditional wide-field fluorescence, SPIM illuminates a living specimen embedded in agar with a single plane of light rather than a traditional cone of light focused to a diffraction-limited spot. Such a light sheet can be swept rapidly across a large three-dimensional structure and the data can be collected via wide-field fluorescence with fast and sensitive charge-coupled device (CCD) cameras. Image acquisition with such a system could provide both a fast throughput and a high signal-to-noise ratio. As the 'maximum' resolution of the illuminating plane is effectively the 'thinness' of that plane, low numerical aperture objectives can be used, providing a large depth of field while still retaining the 'resolution' of the plane. Rotating a sample perpendicular to the detection axis allows the three-dimensional sample to be resliced, producing a high-resolution image with isotropic voxels.

The second technique, described by Dodt *et al.*[9], tackled another potential problem for light-based optical investigation of large volumes of tissue. Biological tissue is highly scattering and the penetration by light (even infrared illumination) is as a result limited to depths less than 1.5 mm. Dodt *et al.*[9], using an old but rarely used technique[10], instead immerse fixed tissue in a medium with a similar refractive index. As this medium pene-trates a sample, differences in the refractive indices of various parts of the tissue become minimized, allowing light a clear passage with little scattering. They then imaged large fixed samples using a simpler light sheet approach than SPIM called 'ultramicroscopy' after the original description of light sheet–based microsopy[11]. This technique involves scanning the sample up or down through a light sheet created by two aligned illumination sources at opposite sides of the sample. Using such a protocol, the authors clear 2-cm specimens, imaging neurons and their fine dendritic extensions within a large volume of brain.

In both cases, reconstructions did not require the splicing together of separate small regions that were imaged separately. Both seem to have sufficient resolution to easily see axonal bundles through considerable depths. The recent advent of transgenic animals that express fluorescent proteins in axons are ideal subjects for this type of technique[12,13]. These imaging techniques will also find useful applications in other fields such as developmental biology, as shown by the authors of both papers with striking reconstructions of whole embryos. Finally, a large digital atlas of the expression pattern of 20,000 genes in the adult mouse cortex was recently completed[14]. That data set required nearly heroic automation of the cutting, collection, staining and individual imaging of thousands of brain sections. Combining these new imaging techniques with multicolor fluorescence–based expression data could make this process much quicker.

Eventually, of course, neuroscientists will want it all: large-volume reconstructions of the brain at nanometer resolution. This task, however, will require techniques that can accommodate multiscale imaging: high resolution for synaptic circuits and lower resolution for pathways. The results of the first projectomes will provide a foundation upon which to map future synaptic resolution 'connectomes'.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

1. Donnert, G. *et al. Biophys J.*; published online 16 February 2007.
2. Gustafsson, M.G. *Proc. Natl. Acad. Sci. USA* **102**, 13081–13086 (2005).
3. Betzig, E. *et al. Science* **313**, 1642–1645 (2006).
4. Rust, M.J., Bates, M. & Zhuang, X. *Nat. Methods* **3**, 793–795 (2006).
5. Denk, W. & Horstmann, H. *PLoS Biol.* **2**, e329 (2004).
6. Verveer, P.J. *et al. Nat. Methods* **4**, 311–313 (2007).
7. Sharpe, J. *et al. Science* **296**, 541–545 (2002).
8. Huisken, J., Swoger, J., Del Bene, F., Wittbrodt, J. & Stelzer, E.H. *Science* **305**, 1007–1009 (2004).
9. Dodt, H.-U. *et al. Nat. Methods* **4**, 331–336 (2007).
10. Spalteholz, W. Über das Durchsichtigmachen von menschlichen und tierischen Präparaten. (S. Hierzel, Leipzig, 1914).
11. Siedentopf, H. & Zsigmondy, R. *Annalen der Physik* **10**, 1–39 (1903).
12. Feng, G. *et al. Neuron* **28**, 41–51 (2000).
13. Scott, E.K. *et al. Nat. Methods* **4**, 323–326 (2007).
14. Lein, E.S. *et al. Nature* **445**, 168–176 (2007).

Do-it-yourself RNAi made easy?

Bernard Mathey-Prevot & Norbert Perrimon

Enzymatically generated small interfering RNAs are a welcome addition in the tool box of scientists performing large-scale RNAi screens.

In 2006, Craig Mello and Andrew Fire received the Nobel Prize for their discovery that long double-stranded RNAs (dsRNAs) trigger the potent and specific mRNA degradation in *Caenorhabditis elegans* via the RNA interference (RNAi) pathway[1]. The subsequent observation by Tuschl and colleagues that small interfering RNAs (siRNAs) could be used to trigger this process in mammalian cells[2] allowed this method to be applied to systematic loss-of-function studies in cell-based assays. This methodological leap turned the production of RNAi reagents into a multimillion-dollarenterprise as many companies devoted considerable efforts to develop products with high specificity and efficacy.

Today the most widely used reagents for genome-scale RNAi studies are chemically synthesized siRNAs or virally encoded

Bernard Mathey-Prevot and Norbert Perrimon are in the Department of Genetics, Howard Hughes Medical Institute, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115, USA.
e-mail: perrimon@receptor.med.harvard.edu

shRNAs (see ref. 3 for a more detailed review on RNAi reagents). Although these have been by-and-large successful, issues of specificity, effectiveness and cost associated with their use have emerged, and the field would benefit from an improvement over the existing reagents used in RNAi screens. In this issue Kittler *et al.*[4], building on their previous work[5], present an alternative to these common RNAi reagents in the form of an enzymatically generated siRNAs library.

Aside from cost considerations, the main drawback of chemically synthesized siRNAs has been their propensity to silence unintended targets, irrespective of the algorithm used in sequence optimization. The chief reason for the additional activity is likely to lie in the seed region, a 6-nucleotide stretch at the 5′ end of the guide strand of the siRNA, which can pair with complementary regions in the 3′ untranslated region of various transcripts, resulting in the siRNA acting as an miRNA[6]. One can potentially avoid this off-target effect by diluting the siRNA, but the dilution required compromises the siRNA on-target efficiency as well. To resolve this quandary, researchers proposed using pools of large numbers of siRNAs, each targeting the same gene. They expected that the individual concentration of each siRNA would be too low to give rise to off-target silencing, but the cumulative concentration of siRNAs would be high enough to result in efficient on-target silencing.

Building on this principle, Kittler *et al.*[4] generated endonuclease-prepared siRNA (esiRNA) pools from long dsRNA templates. They first identified the longest common region for each gene encoding multiple transcripts and subjected these regions—together with cDNA sequences encoding only a single transcript—to DEQOR, a software program that selects 400–600-bp regions predicted to yield siRNAs optimized for their efficacy and specificity. Then, using specific primer pairs, tagged with T7 promoter sequences, they successfully amplified the selected regions from a normalized cDNA library and transcribed the PCR products *in vitro* to yield long dsRNAs, which they then digested with endonuclease to generate short 18–25-bp dsRNAs. After purification and normalization, they arrayed the esiRNAs into 384-well plates (**Fig. 1**). The authors convincingly show that esiRNAs appear as effective in their on-target silencing as optimized siRNAs, but are less prone to off-target silencing than siRNAs or shRNAs. Regarding the issue of off-target effects, which has been a problem plaguing the RNAi technology[3,7], Kittler *et al.*[4] show a promising advantage of esiRNAs. Using microarray profiling, they document that esiRNAs are associated with a significant decrease of the silencing of unintended transcripts when compared to single or small pools of siRNAs designed against the same gene.

Therefore, esiRNA libraries will be welcome news to any laboratory thinking of using RNAi in their studies as they are a cost-effective alternative to chemically synthesized siRNAs and do not require testing of several shRNA constructs to effectively silence a particular gene. Notably, the authors are committed to make esiRNAs an open resource, as the primer sequences used for the human library are already published, those for the rat and mouse libraries soon will be published, and the PCR templates will be made available through the German Genome Resource Center, a nonprofit organization.

Are esiRNAs the magic-bullet reagent for RNAi? First, for cell lines that are difficult to transfect, virally encoded shRNAs will remain the reagent of choice. Second, a few important questions need to be resolved before discarding siRNAs to the recycling bin. Optimal and consistent digestion of long dsRNAs and careful purification of the resulting short dsRNAs are critical to the successful application of esiRNAs, but can be tricky to achieve. Additionally, there is no control over which siRNAs will be present after the enzymatic digestion, a potential concern for reproducibility between different preparations. Of concern as well, we note that the DEQOR algorithm that Kittler *et al.*[4] designed minimizes, but does not completely eliminate, the presence of occasional siRNAs that have perfect homology to other genes. The risk, as reported for *Drosophila melanogaster* screens[7] where long dsRNAs (which are intracellularly converted to siRNAs) are routinely used, is that these few cross-reacting siRNAs might create a source of false positives in large-scale screens. Last, despite the claim that esiRNAs do not trigger an interferon response in HeLa and HCT116 cells, a wider range of cell lines needs to be tested before putting this issue to rest.

To conclude, the development of esiRNA libraries should be saluted as a welcome and notable alternative to existing reagents, either as primary reagents for large-scale screens or for result-validation of siRNA- or shRNA-induced phenotypes. The next step will be to bring them into common use.

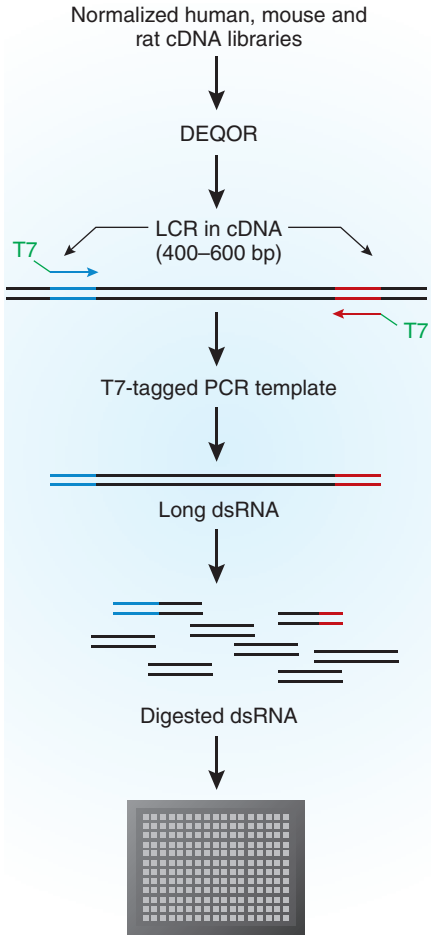

Figure 1 | Creation of an esiRNA library for genome-wide RNAi screens. Appropriate cDNA target sequences, chosen with DEQOR software, are PCR amplified and *in vitro* transcribed. The resulting long dsRNAs are digested with endonuclease and purified to yield a population of uniform short dsRNAs, which are then arrayed in multiwell plates.

COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

1. Fire, A. *et al. Nature* **391**, 806–811 (1998).
2. Elbashir, S.M. *et al. Nature* **411**, 494–498 (2001).
3. Echeverri, C.J. & Perrimon, N. *Nat. Rev. Genet.* **7**, 373–384 (2006).
4. Kittler, R. *et al. Nat. Methods* **4**, 337–344 (2007).
5. Buchholz, F., Kittler, R., Slabicki, M. & Theis, M. *Nat. Methods* **3**, 696–700 (2006).
6. Birmingham, A. *et al. Nat. Methods* **3**, 199–204 (2006).
7. Echeverri, C.J. *et al. Nat. Methods* **3**, 777–779 (2006).