the sample. This generates a multitude of zero-intensity spots or 'donuts', each of which confers super-resolution and is scanned to form a super-resolved image (**Fig. 1**). Importantly, at saturation, the cross-sections of the ~110,000 donuts are effectively circular, and no rotation of the pattern is needed to ensure that resolution is isotropic.

Using this highly parallel super-resolution setup, the researchers were able to image keratin 19–rsEGFP(N205S) expressed in PtK2 cells; part of the cytoskeleton could then be visualized with 80-nm resolution. In another demonstration, the growth of neurites from a neuron expressing the Lifeact-Dronpa-M159T fusion protein was measured over time. Here each super-resolution frame was measured in 2 s, allowing the growth to be monitored with high spatial and temporal resolution.

An important goal in the methodological development of this field is to achieve three-dimensional (3D) super-resolution imaging over a substantial volume and at video rates. Chmyrov *et al.*[7] do not quite achieve this: the frame rates, despite showing major improvement over those seen in previous efforts, are still low, and super-resolution is demonstrated in 2D rather than 3D, although the authors report a commendable *z* resolution of around 580 nm. Other methods such as 3D STORM[8] and biplane fluorescence PALM[9] have been shown to deliver sub-100-nm axial resolution, but the stochastic nature of these approaches results in substantially longer imaging times.

At present, the authors note that it is the camera and the state transition kinetics of the fluorophores that limit the frame rate. Today camera technology is advancing rapidly and is likely to become a nonissue within a few years. On the other hand, the development of more efficient switchable fluorophores represents the key bottleneck. Fortunately, many excellent laboratories are actively on the hunt for better fluorophores. Indeed, with the intense interest in switchable fluorophores for not just RESOLFT but also PALM, STORM, saturated structured illumination microscopy[10] and their derivatives, microscopists can look forward to a future with a wide palette of different fluorophores optimized for nanoscopy. When that happens, wide-field RESOLFT imaging and its variants may well become a commonplace substitute for wide-field fluorescence imaging whenever high-resolution images are required.

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Enhanced dissection of the regulatory genome

Matthew Slattery & Kevin P White

Methods for high-throughput and high-resolution dissection of enhancers in *Drosophila* are described by two independent groups.

Transcriptional *cis*-regulatory modules, or enhancers, are regions of DNA that integrate input from multiple transcription factors to direct precise spatial and temporal patterns of gene expression[1]. As such, enhancers are key components of the regulatory networks driving the developmental programs that shape metazoan life. Despite their importance, enhancers remain difficult to identify and characterize. This difficulty arises in part because enhancer regions are difficult to predict bioinformatically and are often distal to the genes they regulate, making 'enhancer bashing' relatively low throughput, but also because standard reporter assays often remove enhancers from their native context. In this issue of *Nature Methods*, two groups describe experimental approaches addressing problems with both throughput[2] and context dependence[3] that arise with enhancer characterization.

Genome-wide mapping of protein- and histone-DNA interactions and DNA accessibility has provided a starting point for large-scale annotation of enhancers. However, enhancer activities inferred on the basis of transcription factor binding, chromatin modifications and DNA accessibility can be inaccurate and, when accurate, often lack information regarding the enhancer's tissue or cell-type specificity. Therefore, inferences based on genomic data must be followed up with functional assays, preferably assays that provide information regarding tissue-specific enhancer activity. Gisselbrecht *et al.* begin to address this need with a method termed enhancer-FACS-seq (eFS), which increases the throughput of tissue-specific enhancer characterization[2].

The eFS strategy is based on a concept underlying many traditional reporter-gene assays: coincident expression of a reporter gene and a tissue– or cell type–specific marker indicates an enhancer is active in the given subset of cells. However, eFS is highly parallelized and allows for screening of hundreds of enhancers at once (**Fig. 1a**). First, Gisselbrecht *et al.*[2] cloned thousands of candidate enhancers upstream of the *GFP* reporter gene. This library of candidate enhancers was then used to generate thousands of transgenic animals (*Drosophila melanogaster*, in this case), each carrying one genomically integrated enhancer-*GFP* construct and expressing a tissue-specific marker. As opposed to traditional reporter assays, which are usually microscopy based, eFS uses fluorescence activated cell sorting (FACS) to capture cells expressing both the tissue-specific marker and the *GFP* reporter gene. Thus, GFP^+ cells from various tissue populations were collected via FACS. After isolation of the DNA from this population of cells, enhancer regions were PCR amplified and sequenced using next-generation sequencing. With these enhancer 'counts' in hand, enrichment in a given tissue relative to the enhancer's representation in the overall embryo population provides a list of tissue-specific, active enhancers (**Fig. 1a**).

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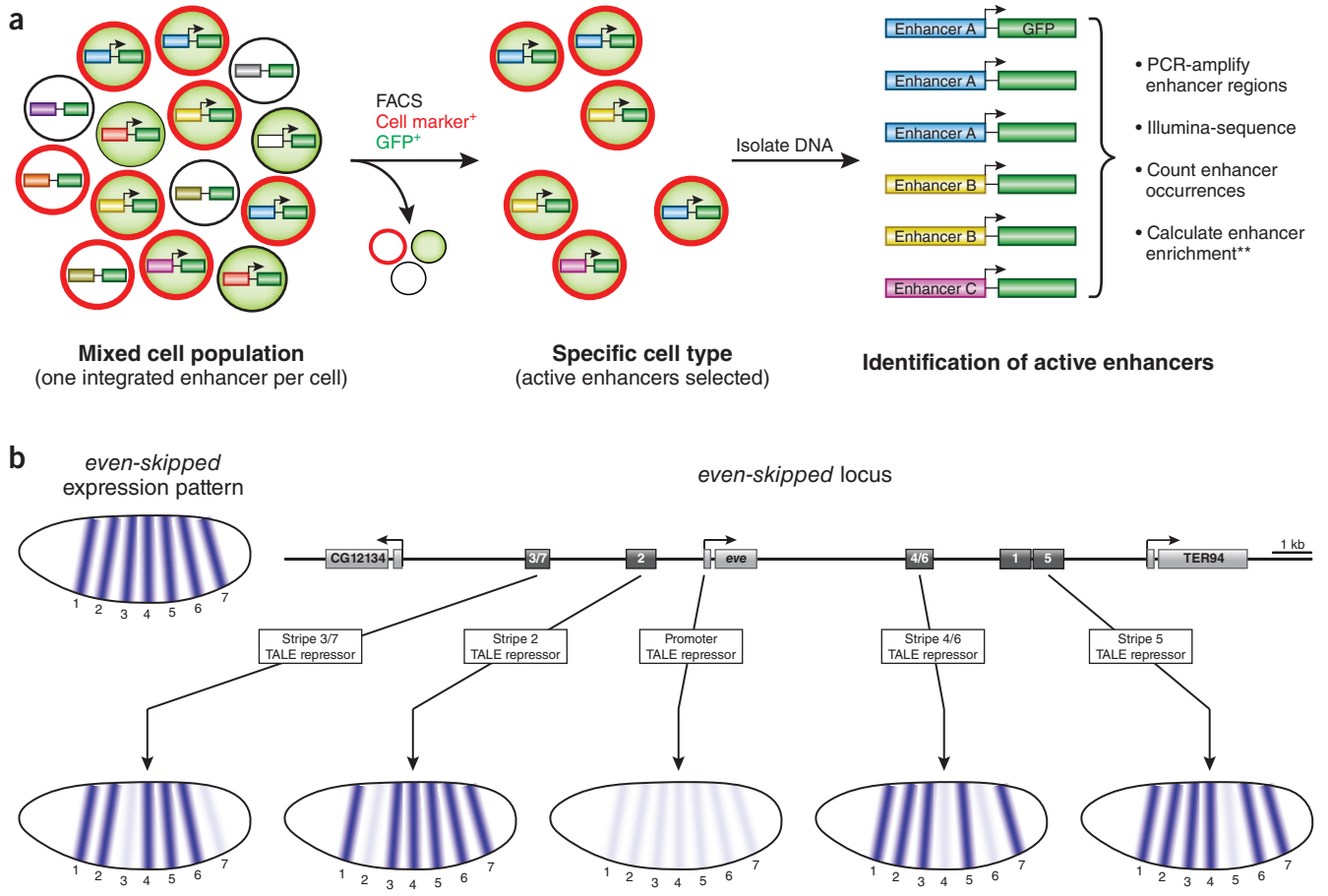

Figure 1 | High-throughput and high-resolution characterization of transcriptional enhancers. (**a**) Workflow of eFS for highly parallel annotation of tissue-specific enhancers. **Enhancer activity (counts) in a sorted cell population is compared to background enhancer counts in unselected cells to identify active enhancers enriched in the sorted population. (**b**) TALE-repressor fusion proteins specifically modulate their target enhancers at the *eve* locus. Modifed from reference 3.

The utility of eFS is clear from the *Drosophila* mesoderm pilot study described by Gisselbrecht *et al.*[2]. eFS analysis of whole mesoderm and mesoderm subsets identified dozens of previously unidentified enhancers, and analysis of DNA motifs in the enhancer sequences implicated both expected and novel transcription factors in the regulation of mesoderm gene expression. Further, a classifier based on the enriched DNA motifs was able to predict mesoderm enhancers with reasonable accuracy, better than could classifiers based solely on DNA motifs of previously characterized mesoderm transcriptional regulators. Although eFS is not a true genome-wide approach, it has a throughput much higher than that of traditional reporter assays while also focusing on tissue-specific expression in an organism rather than in cell culture. As with most assays, cell numbers will be one limitation with eFS; indeed, noise within the data increases in smaller cell populations (that is, mesoderm subsets in comparison to whole mesoderm). eFS-identified enhancers will likely have to be confirmed using more traditional methods to get a true picture of enhancer activity throughout an animal. Nevertheless, as genomic and bioinformatics analyses continue to predict thousands of enhancers, methods such as eFS, streamlined for annotating tissue- and cell-specific enhancers, will be essential.

Although *in vivo* high-throughput annotation of enhancers is an important step toward a mechanistic understanding of gene regulation, enhancer functions must ultimately be understood in the native genomic contexts in which they evolved. In one approach aimed at addressing this problem, Crocker and Stern describe in this issue an elegant method for fine-scale functional dissection of transcriptional enhancers[3]. This approach exploits the activity of transcription activator–like effectors (TALEs), which can be designed to target specific DNA sequences, fused to repressor or activator domains[4]. Focusing on the archetypal 'stripe' enhancers of the *Drosophila even-skipped* (*eve*) locus[5], the authors demonstrate that a TALE repressor (TALER) targeting the *eve* promoter represses all *eve* expression, whereas TALERs targeting specific enhancers repress only the targeted enhancer, leaving the remaining enhancers unaffected (**Fig. 1b**). Beyond demonstrating that individual enhancers can be targeted and modulated using TALEs, which itself is quite interesting, these results indicate that repression of these enhancers is relatively local with no evidence for long-range repression of non–TALER-targeted *eve* enhancers. Further exploration of the mechanisms limiting the spread of repressor activity from one enhancer to another will increase our understanding of the modular nature of enhancers at genes with complex regulatory inputs.

Interestingly, similarly to TALERs, the effects of TALE activators (TALEAs) are also restricted almost entirely to the targeted 'stripe' enhancer, even when TALEAs are ubiquitously expressed[3]. In other words, a TALEA targeting the stripe 3/7 enhancer in all cells increases *eve* expression in only the cells that make up stripes 3 and 7. And a TALEA targeting the *eve*

promoter in all cells increases *eve* expression in only the cells that normally express *eve*. This lack of ectopic expression with TALEAs indicates that cell-specific repressive mechanisms (active repressive inputs or DNA inaccessibility are two possibilities) limit TALEA activity outside of the normal *eve* expression domain.

The beauty of this TALE-based approach is that it is *in situ*; an enhancer need not be removed from its genomic context for a reporter assay[3]. It will be interesting to see whether the properties of the *eve* enhancers, which are among the most studied enhancers in developmental biology, hold true for other regulatory loci. *eve* regulation is an ideal starting point because so much is known about its enhancers, making them easy to target and the results relatively easy to interpret; the same may not be true for other genes. Still, it is clear from these findings at the *eve* locus that TALE-based modulation of enhancers will be a tremendously powerful tool for studying the impact of context—both genomic context and cellular context—on enhancer activity. Although the technologies are nascent, the complementary high-throughput[2] and high-resolution[3] studies described in this issue, in combination with genomic data[6] and additional large-scale studies of enhancer activity[7–10], are beginning to link *Drosophila* genomics to cell-specific gene regulation. These approaches have the potential to greatly inform our understanding of cell-specific regulatory networks not only in *Drosophila* but also across a wide range of species, including our own.

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