halfway through catalytic activation. Compared with the B^{act} complex, several components, such as the RES complex and SF3B6 (a protein subunit of the SF3B complex), are destabilized (positioned at different locations and more loosely associated with the pre-mRNA than before), and the protein PPIL4 is a newly associated component. Several other components were also repositioned.

Strikingly, in B^{AQR}, PRP2 occupies a region 7–13 nucleotides downstream of the branch site, rather than 26–32 nucleotides downstream, as in the B^{act} complex; this reflects the movement of PRP2 along the intron by 19 nucleotides towards the spliceosome core. Moreover, PRP2 is rotated approximately 70° anticlockwise, with certain domains of the protein (the RecA-like domains) adopting the open conformation characteristic of that found after ATP hydrolysis.

A region of PRP2 (the amino-terminal domain) that is not observed in the B^{act} complex exhibits an extended conformation in B^{AQR}, and is organized into three distinct modules. These are referred to as the hook, the clip and the pin. PRP2 interacts with PPIL4 and the protein SKIP through the clip, and together they form a stable 3D assembly that binds to the intron at nucleotides 17–20 downstream of the PRP2 binding site. Moreover, a region of SKIP inserts like a wedge between the two RecA and the HB domains of PRP2, locking PRP2 into an open conformation. Thus, it is proposed that PPIL4 and SKIP form a brake-like structure to stop the movement (translocation) of PRP2.

Translocation of PRP2 leads to a structural change in SF3B1. The HEAT domain of SF3B1, which encloses the branch helix in a closed conformation in the B^{act} complex, exhibits a loose conformation in the B^{AQR} complex, disrupting approximately 60% of SF3B1's interface with the RNA. This alteration to the SF3B1 structure weakens the interaction between the SF3B complex and the protein PRP8 – probably assisting the subsequent release of the SF3A/B complex, which would occur only when Aquarius can catalyse ATP hydrolysis.

An interesting question arises as to how Aquarius coordinates with PRP2 to remodel the spliceosome. PPIL4 and SKIP bind to the intron immediately downstream of PRP2 in B^{AQR}. If PPIL4 and SKIP indeed act as a molecular brake to PRP2, then Aquarius might destabilize the PPIL4 and SKIP bound to RNA, thereby permitting PRP2 movement and thus release of SF3A/B. Alternatively, PRP2 and Aquarius might act sequentially to destabilize the RNA binding of components found downstream and upstream of the branch site, respectively, ultimately liberating the branch site.

In both scenarios, PPIL4 and SKIP need to interact with the intron and PRP2 in a timely manner – only after PRP2 has moved near to the branch site but before it proceeds farther. How the action of PPIL4 and SKIP is temporally regulated is thus a fascinating question.

Saccharomyces cerevisiae lacks Aquarius or PPIL4; instead, its version of PRP2 is responsible for the entire process. So it would seem that PPIL4 and Aquarius co-evolved. Given that PPIL4 is observed only in the B^{AQR} complex, it might not be required for other steps of the pathway. Conceivably, depleting PPIL4 might bypass the need to have Aquarius for splicing. The evolution of PPIL4 and Aquarius in eukaryotes such as humans indicates that another layer of splicing regulation might exist, compared with the situation in yeast. Whether similar regulation also applies to other DEAH-box helicases remains to be studied.

It is a matter of debate whether DEAH-box helicases pull their RNA substrate towards them from a distance or act by stepwise movement (translocation) along the RNA to destabilize their targets during remodelling of the spliceosome[6–9]. The B^{AQR} complex structure provides key evidence supporting the translocation mechanism for PRP2.

A yeast study of Prp16, using the technique of crosslinking, also supports the translocation mechanism[10]. Prp16 was found to crosslink to a region downstream of the branch site when the second splicing reaction was blocked, whereas an ATPase-defective Prp16 mutant crosslinked farther downstream of the branch site – indicating translocation of Prp16 from its anchoring site towards the spliceosome core during the reaction[10]. Further study will be needed to determine whether this represents a shared mechanism for all splicing that depends on DEAH-box helicases.

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Optical imaging

Cocktails of tags enhance microscopy resolution

Alistair Curd

A limit on the resolution of optical-microscopy techniques has been broken by using a mixture of tags to label copies of target molecules in a sample, opening the way to better views of molecular organization in cells. **See p.711**

On page 711, Reinhardt *et al.*[1] report an optical-microscopy technique that can distinguish between tiny objects that are less than one nanometre apart, equivalent to a separation of just several atoms. They use this approach to visualize adjacent points along a DNA double helix and individual proteins found in pairs in cells – accomplishments that other optical-imaging methods would struggle to achieve. The technique could lead to biologists probing the organization of complex arrangements of the molecules of life in greater detail than before, in the native environment of the cell.

For more than 150 years, the builders of optical microscopes have known that there is a limit to the detail that can be seen in an image using conventional techniques, no matter what the magnification. This is because of the way in which light waves spread out after they are confined – by the aperture of a lens, for instance. The resolution limit long prevented scientists from seeing the detailed arrangements of cellular machinery, which works at a scale well beyond that limit. However, over the past couple of decades, researchers have invented ways of working around this limit.

One important group of techniques involves labelling all the copies of a molecule of interest with a tag, and then causing these tags to 'blink', or light up with fluorescence, a few at a time. The positions of the resulting blurred image spots can be calculated much more precisely than when all the molecules are visible at the same time. Over many repetitions of this process, the positions of many of

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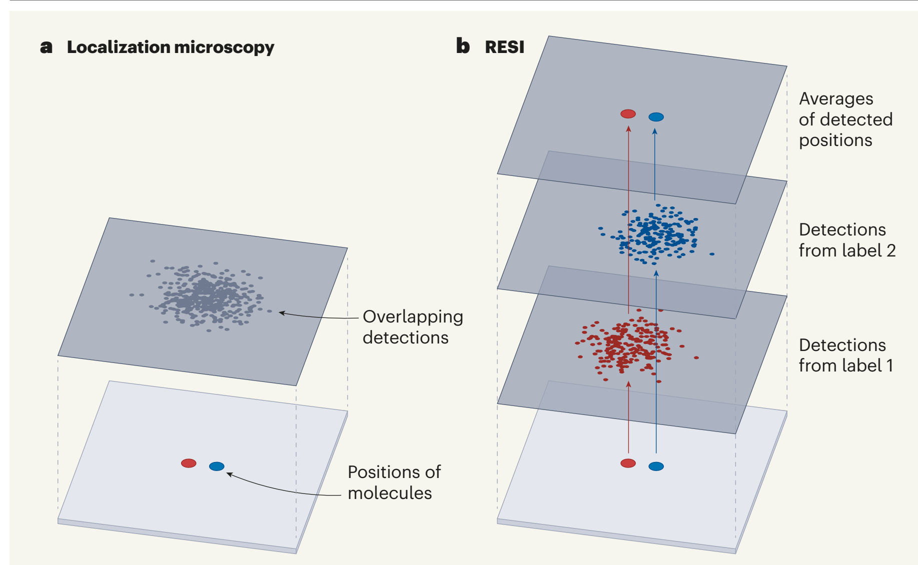

Figure 1 | Resolution enhancement by sequential imaging (RESI). a, In a group of techniques known as localization microscopy, all the copies of the molecule of interest are labelled with tags that light up (blink) with fluorescence, a few at a time. Over many repetitions, the positions of many of the molecules can be found from the average positions of the blinks (each blink is shown as a grey dot). However, blinks from tagged molecules within about 10 to 20 nanometres of each other overlap, making it difficult to distinguish the individual molecules. **b,** In the RESI technique reported by Reinhardt *et al.*[1], adjacent copies of the same target molecule are each labelled with a different tag, enabling the blinks from closely spaced molecules to be differentiated by imaging them in different time periods. Tagged molecules less than one nanometre apart can be distinguished.

the molecules of interest can be found, and their arrangement reconstructed by combining all the precisely determined positions into one data set. This is known as localization microscopy[2].

But even localization microscopy has a precision limit, which is largely determined by the brightness of the blinks. This means that it is still difficult to distinguish between individual molecules, or rather their tags, when they are within about 10 to 20 nanometres of one another. Reinhardt *et al.* have found a way to improve such resolution to less than one nanometre.

In the authors' method, adjacent copies of the molecule of interest are each labelled with a different tag, and blinks originating from the different tags can be clearly distinguished. By collecting multiple blinks from each tagged molecule, the average position of each set of blinks can be determined with much better precision than can be obtained for a single blink — sufficient to locate the two molecules (or rather their tags) at their separate positions. The chosen labelling and imaging method (known as Exchange-PAINT; ref. 3) completely differentiates the blinks from different molecular tags by acquiring them in different time periods (sequentially). It also provides many blinks per tag, thus meeting the two requirements for the technique. The authors name their method 'resolution enhancement by sequential imaging' (RESI; Fig. 1).

Reinhardt *et al.* demonstrate that RESI can discriminate between two tags just a few atoms apart (less than one nanometre) in an engineered structure made of DNA. In natural biological structures in the more challenging environment of the cell, the authors used four different tags to randomly label paired proteins that are about 10 nm apart; this usually (for about 75% of cases) enabled the proteins in each pair to be distinguished and precisely located in three dimensions. Finally, the authors labelled and imaged CD20, a protein targeted by the anticancer drug rituximab.

"The technique can discriminate between two tags just a few atoms apart in an engineered structure made of DNA."

They showed that RESI can provide previously unknown information about how CD20 is arranged in cells and how that arrangement is affected by treatment.

These experiments demonstrate that RESI offers improved resolution when imaging static arrangements of molecules, compared with what can be achieved using other impressive innovations in fluorescence nanoscopy[4]. However, it is not suitable for imaging moving molecules, which requires complementary methods. For example, another localization technique[5], called MINFLUX, comes close to RESI's ability to image detail, but can also precisely track the movement of tagged single molecules, as long as they are at least several hundred nanometres from any other fluorescent molecules[6].

There is still room to improve optical microscopy beyond these new capabilities. The structures observed by RESI must stay still for long enough to get precise information across the field of view — Reinhardt *et al.* report that it takes 100 minutes to find the molecules in a square with dimensions of about 67 × 67 micrometres. For biological experiments, this means that cells must be 'fixed' (preserved in a life-like state), which can introduce some structural distortion and prevents dynamic processes from being visualized over time. The size of the tags is also a limiting factor, because it is the position of the fluorescent part of a tag that is observed, rather than the interesting part of the molecule that the tag is connected to. However, these limitations are common to all high-precision microscopy techniques, and work is under way to address them.

There is a more specific problem for RESI: in samples that require a cocktail of tags to distinguish between molecules (let's say tags A, B and C), the tags become randomly attached to the molecules of interest, which means that nearby molecules in cells will not always be resolvable. For example, the molecules in a pair labelled with A and B would be resolvable, whereas molecules in a pair labelled with A and A — which would often happen by chance — would not. Reinhardt and colleagues explore this issue, and show that using a greater number of distinguishable tags enables molecules to be distinguished more frequently.

It will be fascinating to see what tiny length scales optical microscopy will go on to visualize, perhaps by using greater numbers of distinct, smaller labels. At the same time, there is a need for techniques that image greater volumes in cells than does RESI, but with RESI's level of detail. Work will press on to try to image molecules and their arrangements, not only when they have been stopped in their tracks, but also when they are free to go about their business in cells — thereby revealing ever more about the mechanisms of healthy life and disease at the smallest scale.

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