

Figure 1 | Controlling the reactivity of polyketide chains. **a**, Molecules containing polyketide chains are highly unstable. In the absence of external control, they undergo several reactions, yielding a mixture of products. **b**, Fungal non-reducing, multi-domain iterative polyketide synthase (NR IPKS) enzymes make polyketide-containing compounds as precursors of natural products. The polyketide chains are covalently linked to the enzyme (Enz) and may undergo further modification — in this case, a hexanoyl group (red) is attached at one end of the chain. The resulting product is then guided into the product-template (PT) domain of the enzyme, which folds the polyketide chain into the optimal conformation for subsequent ring-closing reactions. Crawford *et al.*² have obtained crystal structures of the PT domain of a fungal NR IPKS, revealing the mechanisms used by the enzyme to control its substrate, which allow it to faithfully form a single compound, noranthrone, rather than a mixture of products. Me, methyl group.

been cracked wide open in Crawford and colleagues' penetrating account² of the pivotal role of the PT domain. First, the authors find that, although the amino-acid sequence of PksA bears little resemblance to those of FAS enzymes, the crystal structure of the PT domain of PksA has a similar 'double hotdog fold'⁶ to that of certain domains of FAS enzymes — specifically, the dehydrase domains that form part of the FAS machinery responsible for reductive processing reactions. This indicates that the PT domain has evolved from a FAS dehydrase domain, or from its counterpart in bacterial modular PKSs.

The authors also observe a clear binding region for the phosphopantetheine arm of PksA that guides polyketide substrates into the PT domain's binding pocket (Fig. 1b). Furthermore, by obtaining the crystal structure of PksA in which a model substrate is bound,

Crawford *et al.* reveal the presence of a spacious cyclization chamber that can harbour two aromatic rings. Herein lie the two amino-acid residues that initiate the key ring-closing reactions catalysed by the PT domain. Finally, the authors' crystal structures strongly suggest that the binding pocket orientates the polyketide chain ready for catalysis by anchoring the chain's two termini at opposite ends of the pocket.

Crawford and colleagues' analysis² of PT domains from other NR IPKS systems reveals that, within an otherwise highly conserved amino-acid sequence, the most significant level of divergence occurs within the cyclization chamber. This is consistent with the idea that the chambers of different enzymes control the overall configurations of specific substrates prior to reaction, and that they have each evolved to accommodate the correct number of ring-forming reactions necessary to generate a specific product. Taken together, the authors' findings show that the PT domain is the main determinant of chemical architecture produced from NR-IPKS biosynthetic pathways. After the cyclization reaction, the final release of the polyketide product is catalysed by the terminal domain of NR IPKS enzymes, a process that

often results in the formation of another ring in the natural product.

The data presented by Crawford *et al.*², along with those from a recent study of another previously elusive enzyme from a bacterial PKS⁷, bring new clarity to the once murky chemical world of polyketide ring cyclization in natural-product assembly. This fresh insight will certainly provide exciting opportunities for engineering metabolic systems for the preparation of biologically active natural products that have great potential as medicinal agents. ■ David H. Sherman is at the Life Sciences Institute and Department of Medicinal Chemistry, University of Michigan, Ann Arbor, Michigan 48109-2216, USA.

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MICROSCOPY

Light from the dark

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Fluorescence microscopy is the most popular way to image biomolecules, but it leaves many of them in the dark. Non-fluorescent, light-absorbing molecules can now be viewed by a method that turns them into mini-lasers.

What happens when a molecule absorbs a photon from a beam of light? It moves from the ground state to an excited, higher-energy state and then quickly relaxes, giving off the absorbed energy as heat. There are, however, notable exceptions — molecules called fluorophores, which, after some 'wiggling', relax by emitting a lower-energy photon. Employed as molecular tags, fluorophores are invaluable in biomedical microscopy and diagnostics because they render dark molecules visible with high specificity. But what if fluorescent tagging is not an option, as in applications such as endoscopy, and the molecules under investigation stay hidden in the dark? As Min *et al.*¹ report on page 1105, it is still possible to squeeze photons out of such molecules to produce three-dimensional images of biological systems, such as living cells and tissues.

How is this possible? Well, another mechanism for molecular relaxation exists that can be induced by a beam of light. In this process, called stimulated emission, a photon encountering an excited molecule produces a copy of itself, thus adding another photon of the same colour and propagation direction to the beam.

To be effective, the energy of the stimulating photon must match the gap between the excited and the ground state. In fact, the stimulating photons need to be slightly lower in energy than their excitation counterparts, because some of the excited molecule's energy is usually lost as vibrational motion (wiggling) before the photon arrives². Stimulated emission is used to amplify light in lasers³, and to overcome the resolution barrier in fluorescence microscopy⁴. As a molecular process it is almost as effective as light absorption, because both processes depend on the molecule's photon-capture area of about 0.2×0.2 nanometres, which is roughly the area of the molecule itself.

The role of photon-capture area in light absorption and stimulated emission can be thought of as follows. Imagine two synchronized trains of laser pulses directly focused on a molecule: pulses containing excitation photons are followed by pulses of photons for stimulated emission (Fig. 1), with each pulse containing N photons. If one could produce focal light spots that are the size of the molecule, then every photon in the pulses would interact with the molecule — a single photon

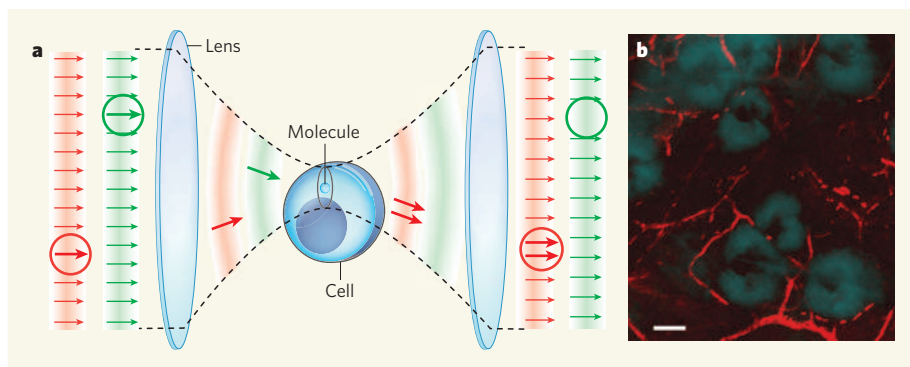


Figure 1 | Imaging non-fluorescent molecules using stimulated emission. **a**, In Min and colleagues' imaging method¹, molecules in a biological specimen are irradiated by ultrashort pulses of light (green). If they absorb a photon, the molecules enter an excited state. The excited molecules are then irradiated by another pulse of light of slightly lower energy (red). When a photon from the second pulse interacts with an excited molecule, the molecule relaxes back to its ground state by emitting a duplicate photon. Overall, a photon from the excitation pulse is absorbed and so is lost (green circles), whereas a photon from the second pulse is doubled up, adding a new, identical photon to the transmitted light (red circles). The gain in photons is measured to quantify the number of molecules in the sample. The colours shown do not correspond to the actual wavelengths of light used in the experiments. **b**, Using their technique, the authors obtained this image of microcapillaries in *ex vivo* mouse skin. The red parts of the image are caused by stimulated emission from haemoglobin in red blood cells. These are overlaid with an image taken using transmitted light, showing sebaceous glands. Scale bar, 50 μm . (Image courtesy of W. Min, S. Lu and S. Chong, Harvard University.)

($N = 1$) from the first pulse would be absorbed and excite the molecule, and another from the second pulse would duplicate itself immediately afterwards.

Unfortunately, because of diffraction, the pulses cannot be focused on a region smaller than about 200×200 nanometres. This area is more than a million times larger than that of a molecule, and so a single photon would most probably miss its target. If, however, more than 1 million photons are used per pulse, one of them will certainly do the job, albeit at the cost of millions of surplus photons that have to be discarded. Discarding surplus excitation photons from fluorescence photons is easily done using filters, and is routine in fluorescence imaging; but singling out the duplicate photons in a stimulating pulse is impossible. Fortunately, to detect the presence of a molecule using stimulated emission, it is sufficient to measure only the number of photons that are added to the pulse — but even that is not straightforward, because N fluctuates from pulse to pulse.

Min *et al.*¹ overcome the challenge of gain measurement by rapidly modulating the excitation beam (and thus the production of duplicate photons), and synchronizing the detection of the slightly strengthened stimulating beam with the modulation^{5,6}. Thus, they reduce the fluctuations in the signal to 'shot noise', which for a train of m recorded pulses amounts to $\sqrt{(N \times m)}$. Because a pulse containing about 1 million stimulating photons yields a new photon from each of n excited molecules in a sample, m such pulses deliver $m \times n$ new photons. The increase in signal should become detectable if $m \times n$ is greater than $\sqrt{(N \times m)}$, which implies that $m > N/n^2$ pulses will make a group of n dark molecules visible. In other

words, detecting 20 molecules requires a minimum of 10,000 pulses. The authors' laser system fires about 10^8 pulses per second, which delivers images of molecules for measurement times of approximately 0.2 milliseconds on a single object point — fast enough for imaging.

Much in this new microscopy contrast mode is determined by the lifetime of the excited state, which is less than 1 picosecond (10^{-12} seconds). The excitation and stimulated-emission photons are preferably squeezed into pulses of about 0.2 ps duration (each providing transient intensities of less than 10 GW cm^{-2} in the sample, which is tolerable for living samples), with the stimulated-emission pulses immediately following their excitation counterparts. This ensures that molecules do not relax before the stimulating photons arrive. Because each round is completed in less than 1 ps, the molecules are almost instantly ready for another round. Indeed, the pulse rate produced by Min and colleagues' laser system is thousands of times slower than the maximum rate that could in principle be used. The recording time required for each measurement could therefore be dramatically reduced by firing pulses at higher rates, which should be facilitated by future developments in laser technology. Increased pulse rates might, however, damage the sample being studied, so the irradiation dose would have to remain at a level compatible with (live) cell imaging.

Another method for imaging non-fluorescent molecules has previously been reported⁵, in which the loss of photons, rather than the gain, is measured when an excited state of the molecules absorbs photons to enter an even higher energy state. The problem with this is that molecules in excited states are reactive, which makes them prone to decomposition.

A strong advantage of Min and colleagues' approach¹ is that the excited molecules are always forced back to the non-reactive ground state. Nevertheless, both methods provide three-dimensional resolution⁷ because their signals stem mainly from molecules in the focal region, which can be raster-scanned through a sample to build up an image. Min and colleagues' method¹ also yields more photons per molecule than stimulated Raman scattering, a phenomenon that has recently also been pioneered by the same group⁶ for imaging non-fluorescent molecules.

But how does the new method compare with fluorescence? Unlike stimulated emission, fluorescence is randomly emitted in space and is thus harder to collect. But for most practical purposes, fluorescence still wins out because the signal can be freed from background noise. However, if non-fluorescent molecules are to be studied and fluorescent labelling is impractical, as is the case in many applications, then the stimulated-emission technique will come into its own.

Stimulated emission has been prominently used in stimulated emission depletion (STED) microscopy^{4,8}, to keep all the fluorophores in a sample dark except for those in a spot smaller than the diffraction resolution limit of the microscope. But in STED microscopy, apart from its ability to switch fluorophores off, there is no interest in stimulated emission per se. Indeed, in related nanoscopy methods⁸, stimulated emission has been replaced by other mechanisms for keeping fluorophores dark, such as flipping the spin of one of their electrons (triplet-state pumping) or relocating some of their atoms (*cis-trans* photoisomerization)⁸. By contrast, in Min and colleagues' technique¹, stimulated emission is the actual goal. An intriguing possibility for the future would be to design a set of laser pulses that fulfil both roles of stimulated emission — switching off molecular signals and stimulating photon emission — to provide images of unlabelled, non-fluorescent molecules at sub-diffraction (nanoscale) resolution for the first time. So, for many reasons, Min and colleagues' method is a bold step towards unveiling details of live cells and tissues that would otherwise be left uncharted. ■

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