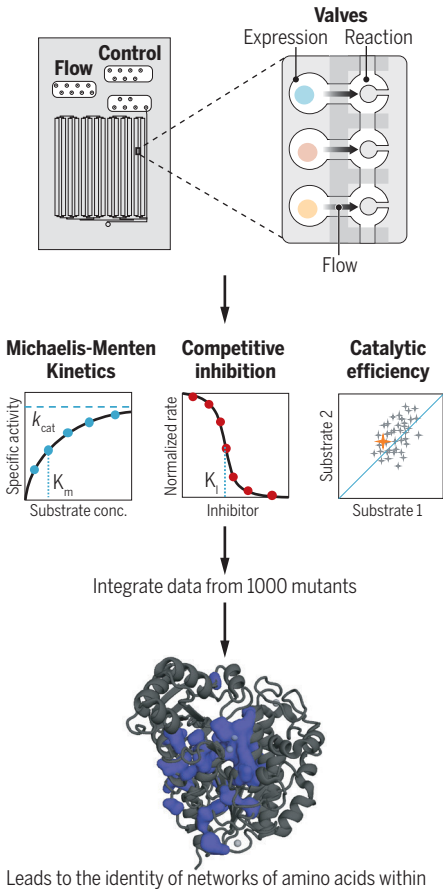
applications in medicinal chemistry and synthetic biology. A new finding of Markin *et al.* is that mutations to PafA are more likely to result in tighter binding than weaker binding of the competitive inhibitor phosphate. This is unusual because mutations on average are expected to decrease binding. It suggests that strong evolutionary mechanisms in enzymes ensure rapid and efficient turnover (catalytic rate) without excessive end-product inhibition.

Although there are limitations in the demonstrations of HT-MEK, there are clear ways to improve generalizing the method and its throughput. HT-MEK requires product formation to be linked to fluorescence. Markin *et al.* used both a non-natural fluorescent substrate and a fluorescent biosensor (*8*) that detects the phosphate product. The biosensor can be used directly for many diverse enzyme classes that produce inorganic phosphate. Probing other enzyme classes will require innovative methods for high-affinity biosensor design in which the product is linked to fluorescence at a response rate faster than that of the internal enzyme kinetics. Also, PafA is a fast enzyme with a catalytic efficiency approximately 10-fold higher than an average enzyme (*9*). Evaluating less efficient enzymes, such as those involved in secondary metabolism, may result in a diminished dynamic range and sensitivity for assay measurements. In addition, Markin *et al.* used a clever experimental design to disentangle protein misfolding from protein activity by performing activity measurements at different temperatures and choosing mutations unlikely to result in global unfolding. PafA is more stable than typical enzymes, and whether this experimental design can be generalized is an open question. Also, the throughput tested by Markin *et al.* was a little more than 1000 mutants, or less than 10% of the throughput of deep mutational scanning. Several technologies can be envisioned to overcome this throughput limit, including parallelization of devices, advances in on-chip oligonucleotide synthesis of entire synthetic genes, and scale down of the reaction chambers.

Beyond exploring the limits of mechanistic enzymology, there are several near-term applications, such as the de novo design of enzymes (*10*). This approach has succeeded in generating active sites of enzymes with atomic resolution, but turnover rates are generally poor without substantial directed evolution; something is missing in the design concept. Teasing apart the mechanistic basis of activity and inactivity for hundreds or thousands of enzyme designs could help identify potentially missing factors. ■

Enzyme dissection at scale

The high-throughput microfluidic enzyme kinetics (HT-MEK) approach allows the simultaneous characterization of more than 1000 single residue mutants of an enzyme. Each chamber expresses a unique mutant that is flowed into an enzyme reaction compartment. Kinetic characterization [such as turnover rate (k_{cat}); Michaelis constant (K_m); and inhibition constant (K_I); and substrate specificity] of each mutant is integrated and mapped back onto the structure of the enzyme, revealing networks of residues involved in different catalytic and functional effects.



Leads to the identity of networks of amino acids within the structure that contribute to specific catalytic mechanisms

REFERENCES AND NOTES

1. B. J. Livesey, J. A. Marsh, *Mol. Syst. Biol.* **16**, e9380 (2020).
2. C. J. Markin *et al.*, *Science* **373**, eabf8761 (2021).
3. C. L. Araya, D. M. Fowler, *Trends Biotechnol.* **29**, 435 (2011).
4. E. Firnberg *et al.*, *Mol. Biol. Evol.* **31**, 1581 (2014).
5. A. Goldenzweig, S. J. Fleishman, *Annu. Rev. Biochem.* **87**, 105 (2018).
6. E. E. Wrenbeck *et al.*, *ACS Synth. Biol.* **8**, 474 (2019).
7. E. E. Wrenbeck *et al.*, *Nat. Commun.* **8**, 15695 (2017).
8. M. Brune *et al.*, *Biochemistry* **33**, 8262 (1994).
9. A. Bar-Even *et al.*, *Biochemistry* **50**, 4402 (2011).
10. D. Hilvert, *Annu. Rev. Biochem.* **82**, 447 (2013).

ACKNOWLEDGMENTS

The authors are supported by the National Science Foundation (NSF) (Chemical, Bioengineering, Environmental and Transport Systems award 2030221 to T.A.W.; NSF Graduate Research Fellowship Program to Z.T.B.) and National Institutes of Health, National Institute of General Medical Sciences under award R21GM129559-01 to T.A.W.

10.1126/science.abj8346

SPECTROSCOPY

Proximity and single-molecule energetics

Scanning probes measure how nearby oxygen molecules affect triplet lifetimes of pentacene

By **Linfei Li** *and* **Nan Jiang**

Probing single molecules in their nanoenvironment can reveal site-specific phenomena that would be obscured by ensemble-averaging experiments on macroscopic populations of molecules. Particularly in the past decade, major technological breakthroughs in scanning probe microscopy (SPM) have led to unprecedented spatial resolution and versatility and enabled the interrogation of molecular conformation, bond order, molecular orbitals, charge states, spins, phonons, and intermolecular interactions. On page xxx of this issue, Peng *et al.* (*1*) use SPM to directly measure the triplet lifetime of an individual pentacene molecule and demonstrate its dependence on interactions with nearby oxygen molecules with atomic precision. In addition to allowing the local tuning and probing of spin-spin interactions between molecules, this study represents a notable advance in the single-molecule regime and provides insights into many macroscopic behaviors and related applications in catalysis, energy-conversion materials, or biological systems.

Single-molecule studies have benefited from the high resolution achieved with well-defined functionalized probes, especially with carbon monoxide–terminated atomic force microscopy (AFM) tips (*2*). The versatility and applicability of AFM have also been enhanced by biasing the tip with gate voltages and supporting molecules on insulating substrates. In this configuration, the conductive AFM tip serves as an atomically controlled charge injector with single-charge sensitivity. Such electrical addressing of electronic states of single molecules (*3*) allows for the study

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GRAPHIC: N. DESAI/*SCIENCE*

of charge distribution and transport in single-molecule devices, organic electronics, and photovoltaics.

Beyond steady-state spectroscopy, excited-state dynamics of single molecules can be measured by using an ultrashort and high-intensity electric (voltage) or optical (laser) pulse (the "pump") to excite the sample. After a nonequilibrium state is generated, a second weaker pulse (the "probe") monitors the change of the excited state. By varying the time delay between the two pulses, the temporal evolution of the excited state can be mapped out.

Peng *et al.* used the electronic pump-probe approach in AFM to measure the lifetime of the excited triplet state of an individual pentacene molecule with atomic precision (see the figure). They observed strong quenching of the triplet lifetime by co-adsorbed molecular oxygen (O_2). The electronic energy-transfer processes had an intriguing dependence on the arrangement of surrounding O_2 molecules, which they controlled by atomic manipulation with the tip. Spin-relaxation measurements of single molecules in space with atomic resolution provide fundamental insights into their local interactions with each other and their nanoenvironment. Such information could be useful in the pursuit of spin-based quantum-information storage and quantum computing (*4*).

Given the radiative relaxation of excited states, SPM-coupled optical spectroscopy provides a powerful tool to perform spatially and energy-resolved spectroscopic studies of single molecules. Specifically, site-resolved excitations of molecules can be induced by highly localized scanning tunnel microscopy (STM) current, and the resulting luminescence, which carries information that describes excited states, can be probed by integrated optical detection systems. This approach revealed redox state–dependent excitation of single molecules and intermolecular excitonic coupling interactions with atomic-scale spatial precision (*5*, *6*). A study of electroluminescence demonstrated selective triplet formation by manipulating electron spin inside a molecule (*7*), which could provide a route to interrogate quantum spintronics and organic electronics at the single-molecule level.

Besides tunneling electrons, the interaction of photons with molecules can provide valuable structural information and chemical identification through measurements of absorption, emission, or scattering of light. In particular, by confining laser light at the atomic-scale SPM junction and taking advantage of plasmon-enhanced Raman scattering, tip-enhanced Raman spectroscopy can overcome the diffraction limit of conventional optical spectroscopy and thereby achieve submolecular chemical spatial resolution (*8*). Such capability provides in-depth insights into single-molecule chemistry and site-specific chemical effects at the spatial limit (*9*).

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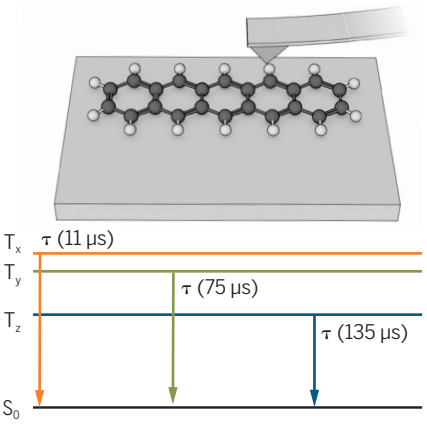
Most excited states induced by photon absorption are incredibly short-lived (on the order of picoseconds to femtoseconds), so time-resolved optical STM techniques have been developed with ultrafast lasers. For example, pump-probe terahertz laser pulses were used to induce state-selective ultrafast STM tunneling current through a single molecule, which allowed the molecular orbital structure and vibrations to be directly measured on the femtosecond time scale (*10*). Optical STM further showed the capability to explore photon and field-driven tunneling with angstrom-scale spatial and attosecond temporal resolution. This experimental platform can be used to study quasiparticle dynamics in superconductor and two-dimensional materials with exceptional resolutions (*11*).

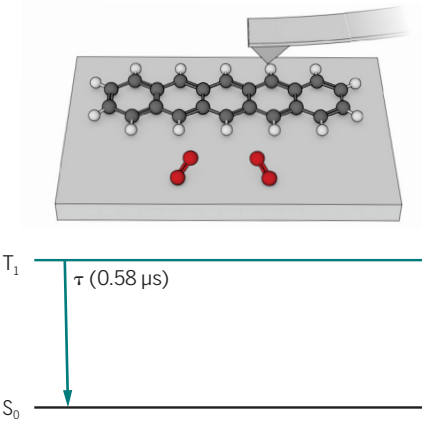
Single-molecule studies could open avenues to access extremely transient states and chemical heterogeneity, such as the vibration of atoms within a molecule, the precession of a spin, ultrashort-lived complex reaction intermediates, and some key stochastic processes of reactions in chemistry and biology. For example, the study of Peng *et al.* relates to the reactivity of electronic excited states of organic molecules to O_2 (and thus air) that affects various natural photochemical and photophysical processes in sunlight, leading to transformation, degradation, or aging (*12*). The insightful descriptions of molecular conformation, dynamics, and function provided by spatially resolved single-molecule studies could inform complex and emergent behaviors of populations of molecules or even cells. ■

Atomically addressing single molecules

Peng *et al.* probed the effect of nearby oxygen molecules on the lifetime of triplet state of individual pentacene molecules on an insulating salt surface.



Molecular interactions
Scanning probe microscopy was combined with electronic pump-probe techniques to follow pentacene excitation. The tip placed oxygen molecules next to pentacene.



Triplet lifetimes
Pentacene exhibits three triplet states, T_x, T_y, and T_z, but nearby oxygen quenches this excitation to one state T_1 with a much shorter submicrosecond lifetime.

GRAPHIC: V. ALTOUNIAN/ *SCIENCE*

REFERENCES AND NOTES

1. J. Peng *et al.*, *Science* **373**, xxx (2021).
2. L. Gross, F. Mohn, N. Moll, P. Liljeroth, G. Meyer, *Science* **325**, 1110 (2009).
3. S. Fatayer *et al.*, *Nat. Nanotechnol.* **13**, 376 (2018).
4. M. N. Leuenberger, D. Loss, *Nature* **410**, 789 (2001).
5. Y. Zhang *et al.*, *Nature* **531**, 623 (2016).
6. B. Doppagne *et al.*, *Science* **361**, 251 (2018).
7. K. Kimura *et al.*, *Nature* **570**, 210 (2019).
8. J. Lee, K. T. Crampton, N. Tallarida, V. A. Apkarian, *Nature* **568**, 78 (2019).
9. S. Mahapatra, L. Li, J. F. Schultz, N. Jiang, *J. Chem. Phys.* **153**, 010902 (2020).
10. T. L. Cocker, D. Peller, P. Yu, J. Repp, R. Huber, *Nature* **539**, 263 (2016).
11. M. Garg, K. Kern, *Science* **367**, 411 (2020).
12. P. R. Ogilby, *Chem. Soc. Rev.* **39**, 3181 (2010).

ACKNOWLEDGMENTS

We acknowledge support from the National Science Foundation (CHE-1944796).

10.1126/science.abj5860