Assembly of multienzyme complexes on DNA nanostructures

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In nature, the catalytic efficiency of multienzyme complexes highly depends on their spatial organization. The positions and orientations of the composite enzymes are often precisely controlled to facilitate substrate transport between them. Self-assembled DNA nanostructures hold great promise for organizing biomolecules at the nanoscale. Here, we present detailed protocols for exploiting DNA nanostructures as assembly scaffolds that organize the spatial arrangements of multienzyme cascades with control over their relative distance, compartmentalization and substrate diffusion paths. The protocol describes the preparation and purification of DNA-conjugated enzymes and cofactors, along with the assembly of these prepared complexes on DNA nanostructures. The architecture of assembled enzyme complexes is then readily characterized using a broad selection of techniques from routine gel electrophoresis to advanced single-molecule imaging. We also describe methods of purifying these nano-assemblies and testing them with functional assays based on either bulk or single-molecule fluorescence measurements. The entire assembly and characterization of a multienzyme complex can be completed within 1–2 weeks.

INTRODUCTION

Biology has evolved complex, multistep enzyme pathways to produce molecules and to harvest energy processes that are vital to the metabolism and propagation of all living systems. The function of these pathways is critically dependent on the relative position, orientation and number of participating enzymes[1,2]. The ability to exert control over such systems on the nanoscale will not only allow us to study the effects of spatial organization on the functions of biochemistry pathways but will also increase our ability to translate biochemical pathways into a variety of noncellular applications, such as high-value chemical synthesis, smart materials, bio-diagnostics and drug delivery. It is thus imperative to develop assembly methods for engineering multienzyme systems, for which their spatial parameters can be easily manipulated on the nanoscale.

Over the past few decades, DNA-based self-assembly has been exploited to construct a multitude of 1D, 2D and 3D nanostructures. The use of double-helical DNA molecules for nanoscale engineering began with Seeman's construction of artificial 'Holliday' junction tiles[3]. The further development of double-crossover (DX) tiles and the use of sticky-end cohesion enabled the creation of periodic nanostructures with distinct topological and geometric features[3–5]. Recent breakthroughs with scaffolded DNA origami[6] and single-stranded DNA (ssDNA) bricks[7,8] have empowered the design and fabrication of a virtually unlimited reservoir of spatially addressable 1D, 2D and 3D nanostructures[9,10], including structures with complex curvatures[11,12], polyhedral meshes[13,14] and periodic DNA crystals[15,16]. Several computational tools, including cadnano[17], CanDo[18] and Tiamat[19], have been developed to facilitate the design of DNA nanostructures, making structural DNA nanotechnology ever more accessible to researchers from other fields.

DNA nanostructures have recently emerged as promising assembly scaffolds for organizing molecules on the nanoscale based on their sequence-driven, programmable self-assembly[5,20–24]. For example, multienzyme cascades can be positioned on DNA nanostructures with precise control over the spatial distances, with the goals of enhancing the mass transport of substrates[25–27], engineering substrate channeling mechanisms[28] and regulating spatial interactions between enzyme pairs[29,30]. Self-assembled DNA nanoboxes and nanocages have been demonstrated for the encapsulation of macromolecular payloads such as antibodies[31,32] and enzymes[33–35]. Tubular DNA nanostructures have been similarly used to construct efficient enzyme cascade nanoreactors[36]. Here we describe protocols for the robust assembly, purification and characterization of multienzyme complexes organized by DNA nanoscaffolds. These protocols have been successfully used to develop a series of artificial enzyme complexes with functions such as facilitated interenzyme substrate diffusion[25], and that include biocatalytic swinging arms[28], compartmentalized enzyme cascades[33] and regulated enzyme nanoreactors[29].

Spatially addressable DNA nanostructures for positioning enzymes

Spatially addressable assembly is one of the most attractive features of DNA nanostructures, due to the well-defined geometry of the DNA double helix and the unique sequence of each oligonucleotide that encodes the overall architecture of the structure. As shown in **Figure 1a**, the B-form DNA double helix has well-defined structural characteristics in aqueous solution, such as a helical diameter of ~2.4 nm, a helical rise of ~3.4 nm for each of 10.5 base pairs (bp) and an ~34.3° rotation angle between two adjacent base pairs[10]. Using DNA nanostructures as the assembly templates, enzymes can be positioned with controlled parameters for spatial distance (**Fig. 1b**)[25] and relative anchor angles (**Fig. 1c**), as well as for the geometric arrangement and stoichiometry of the participating enzymes (**Fig. 1d**)[28]. **Figure 1e–p** shows an example of positioning of a two-enzyme glucose oxidase (GOx)–horseradish

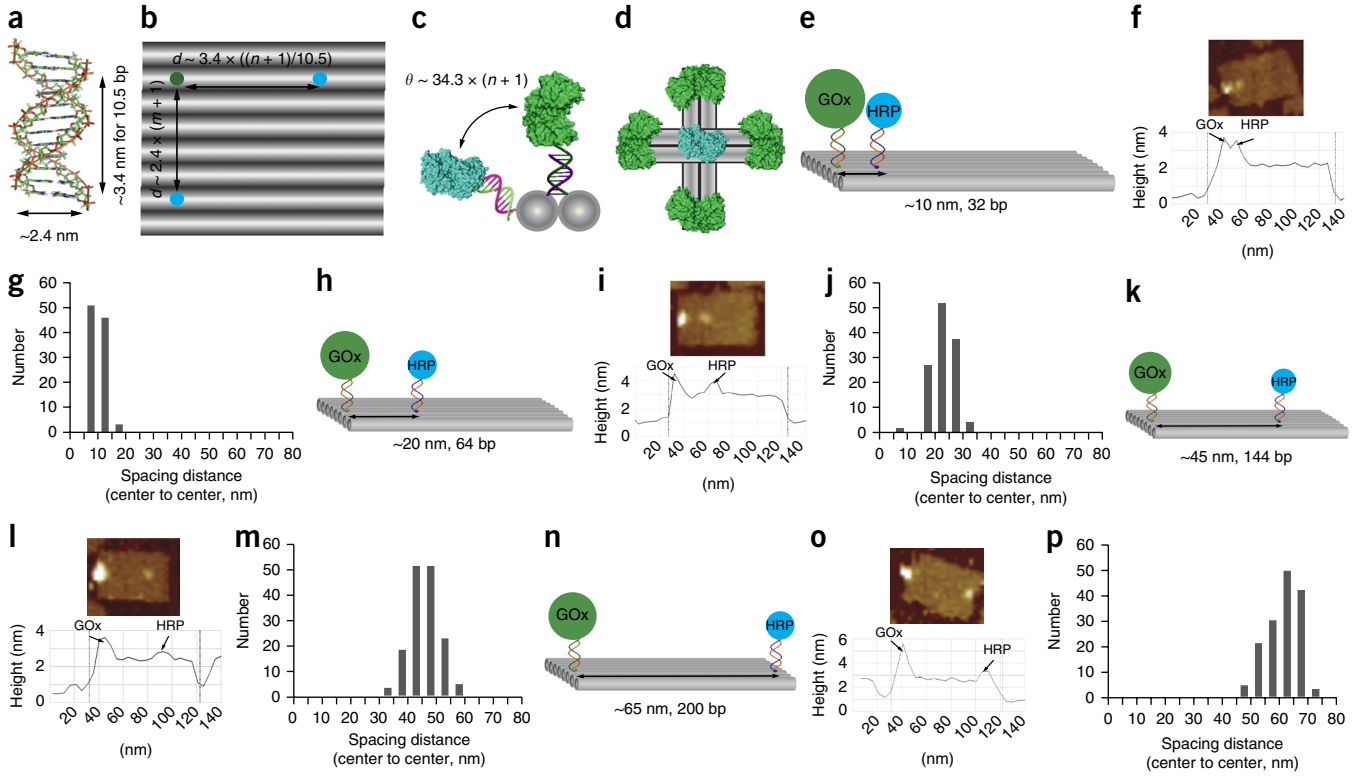

Figure 1 | The assembly of enzymes on spatially addressable DNA scaffolds. (**a**–**d**) DNA double-helix. (**a**) Position of molecules on the DNA nanostructures with controlled (**b**) spacing distances, (**c**) relative angles and (**d**) geometric arrangements. (**e**) The organization of a pair of GOx–HRP enzyme cascade on a rectangular DNA origami tile with 10-nm spacing distance, (**f**,**g**) AFM characterization of the height profile, in which the assembly of two enzymes results in brighter spots due to the increased height as compared with the surface of a DNA origami and (**h**) the statistical analysis of the observed interenzyme distances. The GOx–HRP pair is also assembled and characterized with the spacing distance of (**h**–**j**) 20, (**k**–**m**) 45 and (**n**–**p**) 65 nm on the DNA origami tiles.

peroxidase (HRP) cascade on a rectangular DNA origami with distances ranging from 10, 20, 45 to 65 nm (ref. 25). A analysis of the interenzyme distances (anchor-to-anchor) for the enzyme pair was performed by atomic force microscopy (AFM). The spatial dimensions of a scaffolding DNA nanostructure are described as follows:

- The distance between two anchor positions along the same helix is determined by the number (p) of helical repeats between them:

$$\text{Distance} \sim 3.4\,\text{nm} \times p;\ p = \frac{n+1}{10.5},$$

  where n is the number of spacing nucleotides.
  Note: p must be an integer number for the anchors on the same plane.
- The distance between two anchor positions perpendicular to the helix's long axis can be determined by the number (m) of spacing helices between them:

$$\text{Distance} \sim 2.4\,\text{nm} \times (m+1)$$

- The relative angle between two anchor positions can be controlled approximately by the rotation angle between base pairs:

$$\theta \sim 34.3^{\circ} * (n+1),$$

where n is the number of spacing base pairs. Examples of such angles are as follows: ~100° for two spacing base pairs, ~170° for four spacing base pairs and ~270° for seven spacing base pairs.

Protocol overview

The general strategy for assembling enzyme complexes on DNA nanostructures is illustrated in **Figure 2a**. An enzyme is chemically conjugated to a single-stranded oligonucleotide, which serves as an anchor to hybridize with the corresponding complementary capture strand that is displayed on the surface of a DNA nanoscaffold. **Figure 2b** shows the general flowchart of the assembly, purification and characterization of the multienzyme complexes on the DNA nanostructures. The entire protocol includes the following key steps: (i) the preparation and purification of DNA-conjugated enzymes/cofactors; (ii) the assembly of enzyme complexes onto DNA nanostructures; (iii) the characterization of assembled enzyme complexes; (iv) the purification of assembled enzyme complexes; and (v) the evaluation of activity at the bulk and single-molecule level.

DNA–enzyme/cofactor conjugation and purification

A variety of chemical methods have been developed to link an oligonucleotide with a protein[37], such as aptamer–protein noncovalent interactions[38], N-terminal reactions[39], nickel–nitrilotriacetic acid (NTA)–hexahistidine interactions[40], click chemistry[41], and disulfide[25] and maleimide coupling[26]. We use a simple SPDP (*N*-succinimidyl 3-(2-pyridyldithio) propionate) cross-linker to conjugate the primary amines of the lysine residues on the enzyme surface to a thiol-modified anchor oligonucleotide (**Fig. 3a**). The enzyme solution is first incubated with the SPDP cross-linker, allowing amine-reactive *N*-hydroxysuccinimide (NHS) esters to react with the lysine residues on the enzyme surface.

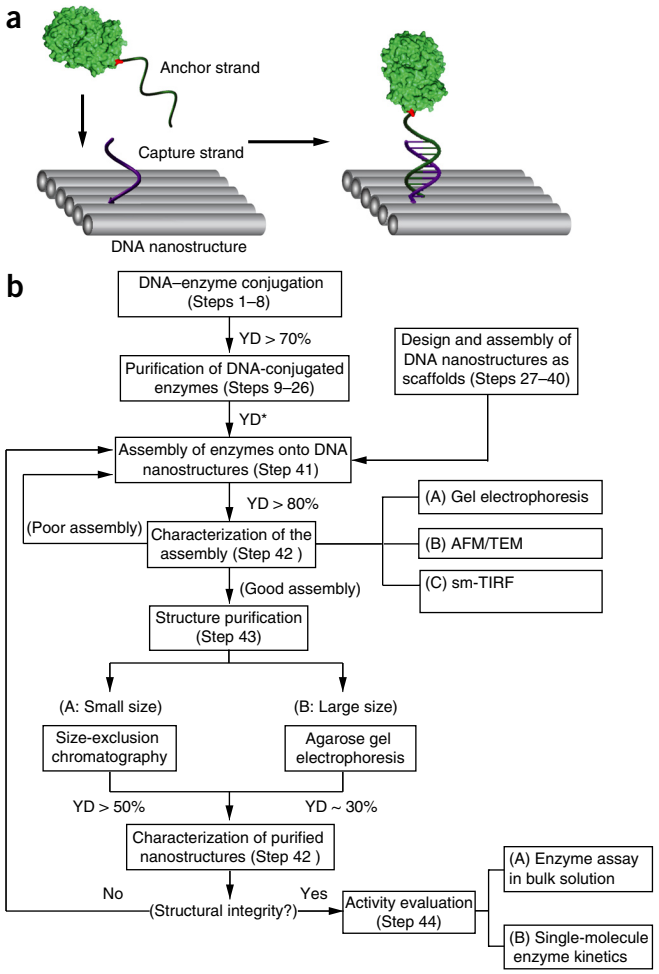

Figure 2 | Overview of DNA nanostructure-scaffolded assembly of multienzyme complexes. (**a**) The assembly strategy for hybridizing a DNA-conjugated enzyme with a complementary capture strand displayed on the surface of a DNA nanostructure. (**b**) The overview of the protocol for the assembly, purification and characterization of multienzyme complexes organized by DNA nanoscaffolds. Corresponding procedure steps and estimated yields are labeled on the workflow. *The purification yield of DNA-conjugated enzymes depends on the collected fraction of enzymes labeled with the exact number of oligonucleotides. The yield may vary from 20% (purified fraction) to >70% (for enzymes labeled with a mixed number of oligonucleotides). AFM, atomic force microscope; TEM, transmission electron microscope; YD, yield.

SPDP-modified enzyme is reacted with a thiol-modified oligonucleotide that undergoes a disulfide bond exchange of the activated pyridyldithiol group.

After the enzyme–DNA conjugation, it is important to completely remove excess unreacted DNA molecules. Centrifugal ultrafiltration with a MWCO filter (e.g., ~30 kDa cutoff) is commonly used as a simple method to filter out smaller DNA molecules from the solution of DNA-conjugated enzymes[25,26,29]. However, DNA molecules tend to bind to the protein surface nonspecifically because of the strong electrostatic interaction between the negatively charged phosphate backbones of DNA molecules and the positively charged residues (e.g., lysine and arginine) on the enzyme surface. Thus, a washing buffer containing a high concentration of NaCl (1.5 M) is used to disrupt this strong electrostatic interaction, and to remove the smaller DNA molecules from the protein surface. As shown in **Table 1**, the low-salt buffer of 10 mM sodium HEPES (pH 7.5) cannot efficiently remove the excess of unconjugated DNA molecules from the DNA-conjugated glucose-6-phosphate dehydrogenase (G6PDHP1). The DNA-to-enzyme ratio is as high as 3.2 after three washes, even though the average SPDP label ratio is only ~0.9. This indicates that excess DNA molecules nonspecifically bind to enzymes without covalent attachment. Conversely, a similar filtration process using a 10 mM HEPES buffer containing 1.5 M NaCl (pH 7.5) reduces the DNA-to-protein ratio to ~0.7, suggesting that most nonspecific DNA molecules are removed. Similarly, the DNA-to-enzyme ratio of a DNA-conjugated lactate dehydrogenase (LDH-P2) is reduced from ~2.7 to ~1.1 by increasing the NaCl concentration from 10 mM to 1.5 M in the washing buffer. The addition of a detergent such as P20 (Surfactant P20) can further disrupt the strong noncovalent interaction between LDH and DNA molecules[42–45], reducing the DNA-to-protein ratio from 1.1 to 0.7 (at a SPDP-to-LDH labeling ratio of ~0.9).

Because of the presence of multiple lysine residues on the surface of enzymes, the collected solution from the centrifugal ultrafiltration is a mixture of enzymes labeled with different numbers of DNA molecules per protein. As shown in **Figure 3b**, anion-exchange fast-protein liquid chromatography (AE-FPLC) is used to isolate a homogeneous population of enzymes labeled with an identical number of oligonucleotides. Using an NaCl gradient increased from 20% (vol/vol) solvent B (~200 mM NaCl) to 55% (vol/vol) solvent B (~550 mM NaCl), a DNA-conjugated G6PDH protein with an average DNA-to-enzyme ratio of ~1.5 is separated into five component peaks, identified as the unmodified G6PDH (~30% B) and G6PDH with 1 (~38–40% B), 2 (~43–46% B), 3 (~48–50% B) and 4 DNA labels (~52% B). The concentrations of the distinct peaks are determined using their A_{260} and A_{280} absorbance values, as shown in **Table 2**. The activities of DNA-conjugated enzymes are measured as shown in **Figure 3c**. The purified DNA-conjugated enzymes are further tested for their assembly onto DNA nanostructures harboring two identical capture strands. As shown in **Figure 3d**, enzymes labeled with one DNA anchor strand may result in a lower assembly yield and the formation of a secondary product with lower mobility in the gel. This formation is likely to consist of two enzymes bound to adjacent capture strands on the same DNA tile. Enzymes labeled with three or four DNA molecules produce aggregated, lower-mobility structures, possibly due to one enzyme bridging two or more DNA tiles. On the basis of these observations, a purified enzyme possessing two identical anchor strands is preferred for assembly onto the DNA nanostructures, as it has been proven to lead to the most efficient assembly, with high yield and reduced aggregation[28]. To achieve better separation, it is recommended to remove excess DNA by centrifugal filtration before AE-FPLC.

DNA molecules can also be modified with cofactors for controlling catalytic function[28,29]. As shown in **Figure 4a**, an amino-modified NAD^+ analog (an enzyme cofactor) is conjugated to an amine-modified oligonucleotide using a disuccinimidyl suberate (DSS) linker on anion-exchange DEAE-Sepharose resin[28,29]. The NAD^+-modified DNA is purified using HPLC and is characterized using mass spectrometry (**Fig. 4b**; **Box 1**).

Assembly of enzyme complexes onto DNA nanostructures

Open-access software is available for designing DNA nanostructures, including Tiamat[19], cadnano[17] and NanoEngineer

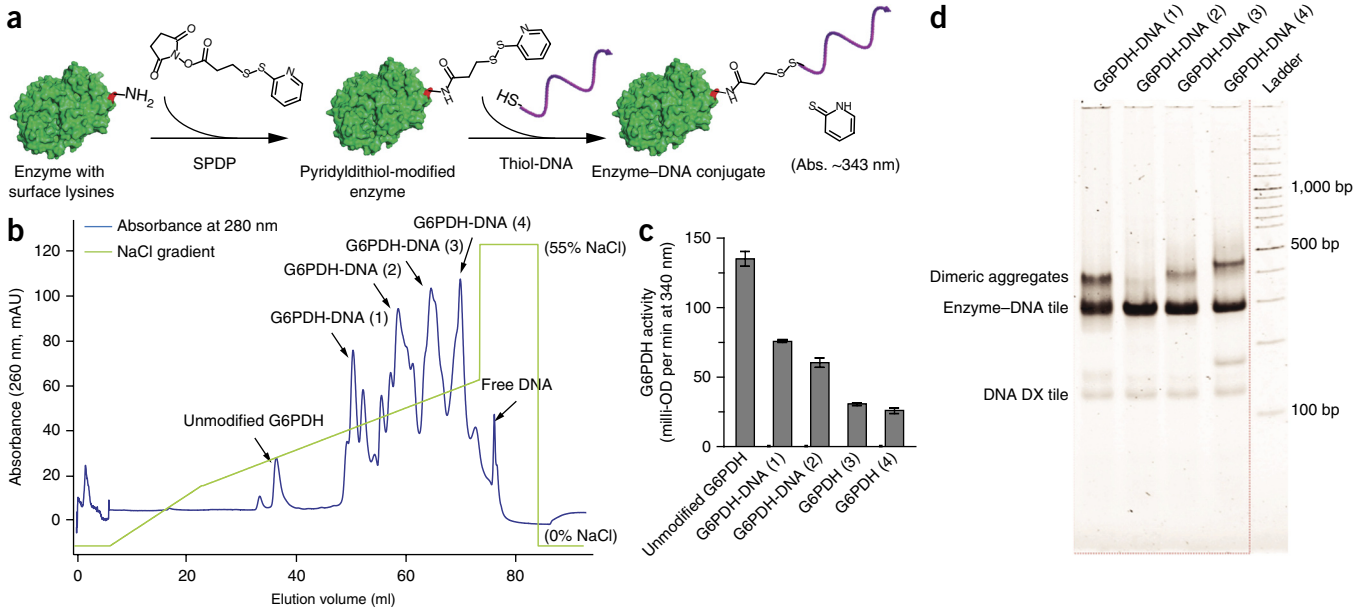

Figure 3 | Chemical conjugation of oligonucleotides with enzymes. (**a**) SPDP chemistry for cross-linking a thiol-modified oligonucleotide to the lysine group of an enzyme. (**b**) Anion-exchange FPLC to purify DNA-conjugated proteins. The enzymes (G6PDH) labeled with different DNA numbers are separated into distinct peaks that are collected in fractions. Conditions: buffer A, 50 mM sodium phosphate (pH 7.5); buffer B, 50 mM sodium phosphate, 1 M NaCl (pH 7.5). The identities of the distinct peaks are assigned using 260-nm and 280-nm absorbance, as shown in **Table 2**. (**c**) DNA-conjugated G6PDH labeled with DNA nos. 0, 1, 2, 3 and 4, collected from FPLC. Assay conditions: 2 nM enzyme is incubated with 1 mM G6P and 1 mM NAD^+ in 100 mM HEPES (pH 8). Enzyme activity is measured by the initial velocity of increased absorbance at 340 nm due to the reduction of NAD^+ to NADH. Error bars represent the standard deviation of three replicates ($N = 3$). (**d**) Native PAGE characterization of the assembly of a series of DNA-conjugated G6PDH proteins onto a DX tile. A threefold molar excess of enzyme was used for the assembly. The gels were stained with SYBR Green I to reveal the mobility of the assembled DNA structures. Abs., absorbance; mAU, milli-optical density.

(Nanorex)[28]. For details, please see previously published articles on designing DNA nanostructures[18,46]. A 2D DNA origami nanostructure is assembled by incubating a 7,249-nt M13mp18 ssDNA scaffold with a set of hundreds of short staple strands using a thermal annealing program, as shown in **Supplementary Figure 1**. As shown in **Table 3**, 2D DNA nanostructures are self-assembled by incubating all oligonucleotides together in 1× TAE–Mg^{2+} buffer following a temperature gradient from 90 to 4 °C over 10 h (refs. 25,28,29,33). For 3D DNA nanostructures such as cages, a longer annealing program is used to slowly decrease the temperature from 80 to 4 °C over 20 h. After the assembly, excess shorter DNA staple strands can be removed by centrifugal ultrafiltration with a 100-kDa MWCO filter. Several DNA nanostructures used for organizing enzyme assembly are shown in **Supplementary Figure 2**. The sequence designs for the DNA nanostructures used in this protocol are available in the **Supplementary Data 1** and should be opened using Tiamat or cadnano.

To efficiently assemble enzymes onto DNA nanostructures, two identical capture strands are displayed on the surface of the DNA scaffolds with a sequence complementary to the anchor strands conjugated to each enzyme. The cooperative hybridization between each two capture and two anchor strands often significantly increases the yield and stability of the assembled enzymes on the DNA nanostructures. Generally, excess enzyme (e.g., threefold molar excess) is incubated with the DNA nanostructure in 1× TAE–Mg^{2+} buffer (pH 7.5). The mixture is temperature-regulated using a PCR thermocycler with a temperature gradient from 37 to 10 °C as follows: 37 °C for 5 min; 36 to 10 °C, 2 min per degree decrease. The assembled sample is then kept at 4 °C for storage.

Characterization of assembled enzyme complexes

Multiple methods have been developed to analyze and visualize the assembled 2D and 3D enzyme complexes, including gel electrophoresis, AFM, transmission electron microscopy (TEM) and single-molecule fluorescence measurement.

Gel electrophoresis. For smaller DNA tiles of a few hundred base pairs in size (e.g., DNA DX tiles), native PAGE (**Box 2**; **Table 4**) is

TABLE 1 | The effect of the salt and detergent concentration on removing nonspecific DNA molecules from DNA-conjugated enzyme solutions.

DNA-conjugated enzyme	SPDP label ratio	DNA-to-enzyme ratio		
		10 mM HEPES (pH 7.5)	10 mM HEPES + 1.5 M NaCl	10 mM HEPES + 0.05% (vol/vol) P20
G6PDH-P1	0.9	3.2	0.7	—
LDH-P2	0.9	2.7	1.1	0.7

Labeled DNA: P1, TTTTTCCCTCCCTCC; P2, TTTTTGGCTGGCTGG. 0.05% (vol/vol) P20 is needed to disrupt the strong nonspecific binding of DNA with LDH[42–45].

TABLE 2 | Quantification of the concentration and DNA labeling ratio of the purified DNA-conjugated G6PDH in **Figure 3** by the absorbance at 260 and 280 nm using equations 4 and 6.

Peak	DNA (TTTTTCCCTCCCTCC)		Enzyme (G6PDH)		Measured absorbance			DNA-conjugated G6PDH	
	ε_{260}	ε_{280}	ε_{260}	ε_{280}	A_{260}/A_{280}	A_{260}	A_{280}	DNA-to-enzyme ratio	Enzyme conc. (μM)
G6PDH (1)	120,000	91,000	62,000	120,000	0.86	1.28	1.49	1.08	6.88
G6PDH (2)					0.96	6.651	6.90	1.89	23.80
G6PDH (3)					1.03	8.557	8.30	2.80	22.29
G6PDH (4)					1.08	6.855	6.34	3.88	13.47

ε_{260} and ε_{280} are expressed per M per cm.

an efficient tool for characterizing the assembled structures[28,29]. As shown in **Figure 5a**, the coupling of an enzyme to a DNA tile results in a clear band shift with lower mobility because of the increased mass and the change in net charge of the enzyme-assembled DNA nanostructure. To improve the imaging contrast, dual-color fluorescent gel imaging is used to confirm the co-assembly of dye-labeled enzymes onto the DNA nanostructures. The assembly yield of enzyme complexes can be approximately estimated by comparing the pixel intensity of a migrated band for an enzyme-assembled DNA structure with the pixel intensity of a migrated band containing only DNA nanostructures.

As shown in **Figure 5b**, the assembly of large DNA nanostructures such as a DNA half cage and a DNA full cage (i.e., DNA origami) can be characterized using agarose gel electrophoresis (AGE; **Box 3**). Unlike native PAGE for characterizing small DNA tiles, the assembly of enzymes onto DNA origami structures produces very small band shifts due to the almost negligible mass of enzymes (a few hundred kilodaltons) as compared with the large DNA origami structures (~4.7 million kDa). It is thus difficult to differentiate between enzyme-assembled DNA structures and the enzyme-free DNA structures using only the band shifts of AGE. The assembly of enzymes onto large nanostructures can be confirmed by using fluorescently labeled enzymes. However, the yield of the assembly cannot be accurately determined by the fluorescent gel.

AFM. For giant, planar DNA nanostructures, such as rectangular DNA origami, AFM (**Box 4**) is used to directly visualize the assembly of enzymes on the surface of the nanostructure, where the assembled enzymes result in brighter spots because of their increased heights on the surface of the DNA origami tile (**Fig. 5c**; **Supplementary Fig. 3**). With AFM imaging, multiple spatial parameters such as interenzyme distance, height and geometric arrangement can be accurately evaluated.

TEM. For 3D DNA nanostructures, negative-staining TEM (**Box 4**) is used to visualize and characterize the assembly or encapsulation of enzymes on/within these 3D nanostructures. As shown in **Figure 5d**, the encapsulation of two enzymes within a DNA nanocage is identified by TEM imaging, where the presence of the enzyme produces a brighter and lighter spot than the DNA structure alone. This is because the enzyme is negatively stained and absorbs fewer electrons (leading to more transmitted electrons) than the uranyl-stained DNA helices that absorb or scatter more electrons (fewer transmitted electrons). However, negative-staining TEM generally produces a low-resolution image of enzyme complexes, which makes it difficult to quantitatively evaluate the encapsulated enzymes.

Single-molecule fluorescence microscopy. For more quantitative analysis of the enzymes encapsulated by 3D DNA nanostructures, we use single-molecule total internal reflection

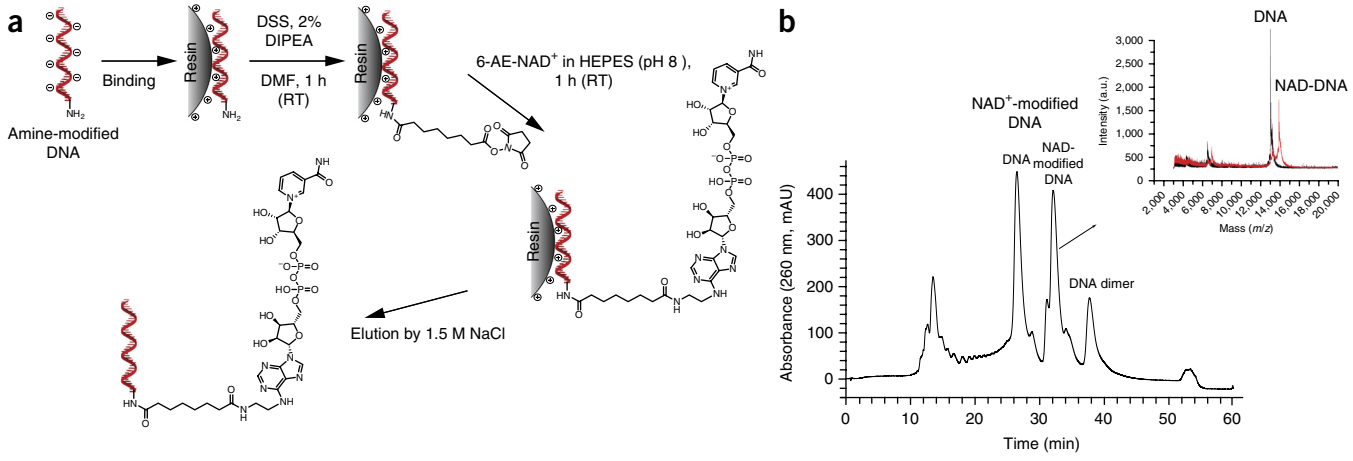

Figure 4 | Chemical conjugation of oligonucleotides with cofactors. (**a**) Reaction pathway for conjugating an aminoethyl NAD+ molecule to a 5′ amine-modified DNA strand using the resin-based DSS cross-linking chemistry. (**b**) HPLC purification and MS characterization (inset, upper right) of NAD+-modified oligonucleotides. MS, mass spectrometry; RT, room temperature (~25 °C).

Box 1 | MALDI-TOF characterization of NAD+-conjugated DNA ● TIMING ~0.5 h

MALDI-TOF is used to characterize NAD+-conjugated DNA molecules by their mass. Unconjugated DNA molecules, linker DNA, NAD+-DNA and DNA dimers are identified by their mass differences, as shown in **Figure 4b**.

Procedure

1. Prepare a saturated solution of 3HPA by adding ~0.1 g of 3HPA to 1 ml of dI H_2O. Ultrasonically mix the solution for 1 min to obtain a saturated solution.

! CAUTION Store 3HPA solid in a dry desiccator for up to ~1 year.

2. Prepare HPLC-purified DNA in dI H_2O. Add 0.5 µl of DNA solution to a clean MALDI sample plate.
3. Add ~2 µl of saturated 3HPA solution to 0.5 µl of HPLC-purified DNA solution.

▲ CRITICAL STEP The final DNA concentration should be approximately a few micromoles to 20 µM. A higher DNA concentration may suppress the mass spectrometry signal.

4. Dry the matrix solution under room temperature (~25 °C) for 20–30 min, or under a vacuum desiccator for 0.5 min.
5. Switch MALDI-TOF to negative mode.
6. Set 'Pulsed Ion Extraction' to 100 ns. Set 'Mass Suppress' to 2,000 Da.
7. Choose an appropriate mass detection window for the target DNA molecules. For example, a 4- to 40-kDa window is used for detecting a 15-kD DNA.
8. Tune the laser intensity to 50%. Set the acquisition frequency at ~100 Hz, average for 1,000 shots.
9. Click the 'Start' button to collect the spectrum.

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fluorescence (smTIRF) microscopy (**Box 5**)[28,47] to characterize the fluorescence colocalization of a Cy3-labeled enzyme and a Cy5-labeled nanocage (**Fig. 5e**)[33]. The average encapsulation yield of an enzyme within a DNA nanocage is estimated by comparing the number of molecules containing the colocalized Cy3/Cy5 signals ($N_{coloc.}$, representing both enzymes and cages) with the number of molecules containing Cy5 signals (N_{cy5}, representing DNA cages). To further evaluate the copies of enzymes encapsulated per DNA nanocage, we use single-molecule fluorescence photobleaching (SMPB) to count the number of photobleaching steps of Cy3 fluorophores that are labeled on DNA-conjugated enzymes (**Fig. 5f,g**). Please see experimental design for detailed methods and analysis.

Purification of enzyme-assembled DNA nanostructures

To analyze the function of the assembled enzyme complexes accurately, it is necessary to remove the impurities of excess and free enzymes, oligonucleotides and aggregations. Two purification methods have been developed to purify enzyme-assembled DNA nanostructures, depending on their size. For smaller (<1,000 kDa) enzyme–DNA tiles (e.g., DX tiles), size-exclusion (SE) chromatography is an efficient approach for separating the different assembly components based on size. As shown in **Figure 6a**, a DX tile–organized multienzyme complex is purified using SE-FPLC, in which the large aggregates are eluted out first, followed by the assembled enzyme complex. The smaller and excess enzymes or DNA fragments are eluted last. These purified components are collected and characterized by native PAGE (**Fig. 6b**).

For larger nanostructures (e.g., DNA origami) that are beyond the separation range of SE chromatography, AGE (**Box 3**) is used to purify the enzyme-assembled DNA structures and to remove excess free enzymes[48]. It is important to check the integrity of the purified nanostructures using native PAGE, AFM or TEM before evaluating their activities.

TABLE 3 | The temperature gradient program for assembling DNA tiles, and 2D and 3D DNA origami structures in 1× TAE-Mg^{2+} buffer.

DNA tile/2D DNA origami (~10 h)		3D DNA origami cage (~20 h)	
Temperature (°C)	Gradient	Temperature (°C)	Gradient
90	30 s	80	2 min
86–71	1 min/step	65–25	30 min/step
70–60	10 min/step		
59–30	15 min/step		
29–26	10 min/step		
25	25 min		
4	Hold	4	Hold

1× TAE–Mg^{2+} buffer = 40 mM Tris, 20 mM acetic acid, 2 mM EDTA and 12.5 mM magnesium acetate, pH 8.0.

Activity evaluation of assembled multienzyme complexes

Colorimetric or fluorescence assays are commonly used to evaluate enzyme activities in bulk solution, where enzyme-catalyzed reactions produce a change in the absorbance of the solution or release a fluorescence signal that is proportional to the concentration of product molecules. As shown in **Figure 7a**, the activity of a DNA nanocage–encapsulated G6PDH complex (cage-[G6PDH]) is evaluated by monitoring the increased absorbance at 340 nm due to the conversion of NAD^+ to NADH by the oxidation of glucose-6-phosphate. In a typical time-course assay, the fitted value of the initial slope is used to characterize the initial velocity of the enzyme-catalyzed reaction at the starting substrate concentration. Because excess free enzymes are removed from the cage-[G6PDH] solution, the activity enhancement of cage-[G6PDH] is estimated by directly comparing the initial velocity of the encapsulated enzyme with the value of the nonencapsulated enzyme at the same enzyme concentration. Further, Michaelis–Menten kinetic analysis is performed by measuring the speed of the reaction, depending on a series of substrate concentrations. As shown in

Box 2 | Native PAGE characterization of the enzyme assembly ● TIMING 6–8 h

Native PAGE is used to characterize the assembly of small DNA structures and the assembly of enzymes. Native PAGE separates DNA nanostructures depending on their structural size, shape and charge. As shown in **Figure 5a**, the addition of an enzyme to a DNA tile results in a clear band shift with slower electrophoresis mobility because of the increased mass of the structure. The pixel intensity of the labeled bands (such as EB or SYBR dyes) can be used to estimate the assembly yield of the enzyme–DNA nanostructures, using the following equation:

$$\text{Yield} \sim \frac{\text{Intensity}_{\text{enzyme–DNA complex}}}{\text{Intensity}_{\text{enzyme–DNA complex}} + \text{Intensity}_{\text{DNA structure alone}}}$$

The native PAGE solutions (0–20%) are prepared by mixing 40% acrylamide solution and 1× TAE–Mg^{2+} buffer solution (**Table 4**). The percentage is decided based on the size of the assembled structures. For DNA structures larger than 1,000 bp, such as DNA origami, AGE, with a larger pore size, is used to characterize the assembly of the structures.

Procedure

1. The 3% (vol/vol) native PAGE is prepared by mixing together 3 ml of 40% acrylamide solution, 4 ml of 10× TAE–Mg^{2+} stock solution and 33 ml of dI H_2O.

! CAUTION PAGE solution is toxic. Acrylamide:bis-acrylamide solution is highly toxic and is a potential human carcinogen, teratogen and neurotoxin. Wear protective gloves and clothes.

2. The polymerization of the gel is initiated by adding 263 µl of APS and 14.7 µl of TEMED to 35 ml of the gel solution. The mixture is immediately loaded into the gel cassette (SE 600 Vertical Unit, GE Healthcare) using a 50-ml transfer pipette.

▲ CRITICAL STEP Make sure that there are no air bubbles trapped inside the gel after loading into the gel cassette.

3. Insert a comb with 15 wells. The gel-casting assembly should be left on the bench at room temperature for ~3 h for the polymerization.

■ PAUSE POINT The polymerized gel can be stored at 4 °C for up to 1 month. It is recommended to wrap the gel cassette with plastic film to avoid dehydration.

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4. Place the gel cassette into the electrophoresis tank (filled with 1× TAE–Mg^{2+} buffer solution), and set the temperature of the water circulator to 15 °C.

▲ CRITICAL STEP Control of the temperature for PAGE is important for accurately analyzing the assembly of structures. A low temperature promotes the aggregation of the structures, whereas a temperature higher than the DNA melting point may induce the thermal dissociation of the structures.

5. Add 1 µl of 10× nondenaturing loading dye to 10 µl of DNA sample, with the final concentration of DNA tiles at ~50–200 nM. Load 10–20 µl of sample into each well.

6. The gel electrophoresis should be run at a constant voltage of ~200 V for 2–3 h, depending on the size of the DNA structures.

! CAUTION High voltage.

7. After the electrophoresis, the gel is stained in ~100 ml of SYBR Green I/Gold solution (1:10,000 dilution) for 10 mins, followed by rinsing with ~200 ml of dI H_2O once.

▲ CRITICAL STEP SYBR Gold stains both single- and double-stranded DNA, as well as proteins. SYBR Green I preferentially stains double-stranded DNA.

8. Image the gel on a UV transilluminator with the SYBR gold/green I channel selected.

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Figure 7b, a detailed kinetic analysis shows that the enhancement of a cage-[G6PDH] is mainly attributed to the boosted turnover number of the enzyme (approximately fivefold increase in k_{cat}), rather than the change of K_m.

As we recently demonstrated[28,33,49], smTIRF can be a useful technique for characterizing the activity of assembled enzyme complexes at the single-molecule level. Compared with bulk approaches that measure the average behavior of a bulk population, single-molecule fluorescence enables the direct observation of catalytic turnovers for individual enzymes. As shown in **Figure 7c**, we use a NADH–phenazine methosulfate (PMS)/resazurin–coupled reaction to convert a nonfluorescent resazurin to a strongly fluorescent resorufin for detecting the activity of G6PDH, for which the spikes of fluorescence signal indicate the repetitive turnovers of substrates catalyzed by individual enzymes[33,49]. As expected, more frequent fluorescent spikes are observed with the addition of enzyme substrate as compared with a control background without the addition of substrate (**Fig. 7d**). Compared with nonencapsulated enzymes, an enhanced turnover of DNA cage–encapsulated enzyme is observed. That is, the average spike frequency is increased by ~1.6-fold for a cage-[G6PDH] as compared with nonencapsulated G6PDH[33]. smTIRF also reveals the fractionof active enzymes for which inactive enzymes show no or very few fluorescence spikes. This information cannot be obtained using traditional bulk assays. From these single-molecule studies, mechanistic details of enzyme activity can be obtained. For example, our studies show that a DNA nanocage not only boosts the turnover number of the encapsulated enzyme but also increases the fraction of active enzymes in solution. Furthermore, the single-molecule fluorescence measurement can be used to detect stochastic fluctuations of enzyme turnover rates via transient spikes

TABLE 4 | The preparation of 40 ml of 3–20% native PAGE with the suggested ranges of separation

Gel percentage	3%	5%	8%	12%	15%	20%
40% Acrylamide (ml)	3	5	8	12	15	20
10× TAE–Mg_2^+ (ml)	4	4	4	4	4	4
H_2O (ml)	33	31	28	24	21	16
Separation range (bp)	100–1,000	75–500	50–400	35–250	20–150	5–100
Bromophenol blue (bp)	100	65	15	20	15	15
Xylene cyanol (bp)	460	260	60	70	60	45

Bromophenol blue is used as a high-mobility marker dye, and xylene cyanol is used as a low-mobility marker dye.

in intensity from the generation of the fluorescent product resorufin. Such fluctuations in activity have been previously observed for various enzymes[43–45] and are thought to be induced by conformational switching between more and less active substates[45–47].

Applications of the protocol

The combination of spatially addressable DNA nanostructures with enzyme–DNA conjugation chemistry offers great opportunities for engineering artificial multienzyme complexes. Applications may include control over spatial parameters between

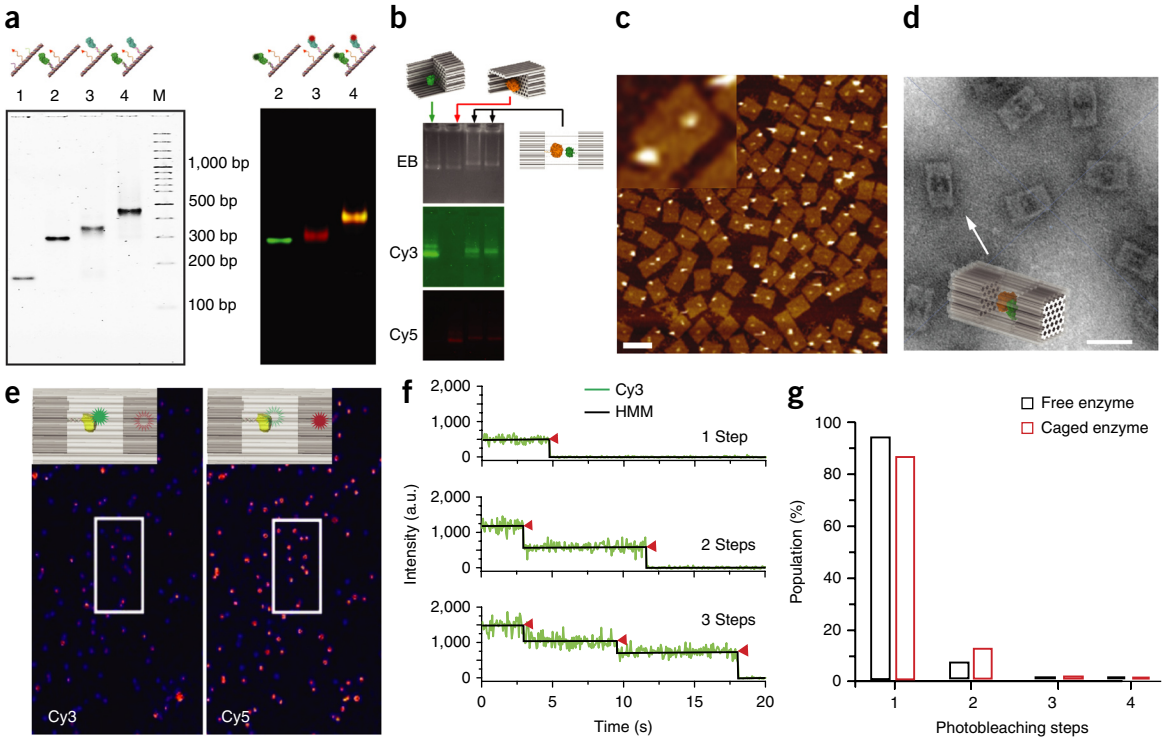

Figure 5 | Characterization of enzyme-assembled DNA nanostructures. (**a**) Native PAGE (3% (vol/vol)) characterization of a series of nanostructured complexes consisting of a pair of G6PDH–MDH molecules. Left: SYBR Green I–stained gel of (1) a DNA tile with a NAD^+-modified arm; (2) a G6PDH-NAD^+ semistructure; (3) a MDH-NAD^+ semistructure; and (4) a fully assembled G6PDH-NAD^+-MDH structure. Right: fluorescence gel image of an Alexa 555–labeled G6PDH-NAD^+ structure (lane 2), an Alexa 647–labeled MDH-NAD^+ structure (lane 3) and a dual-color image of a G6PDH (Alex Fluor 555–labeled)-NAD^+-MDH (Alexa Fluor 647–labeled) structure (lane 4). M: double-stranded DNA ladder[28]. (**b**) AGE characterization of a DNA nanocage encapsulating a GOx–HRP pair[33]: lane 1 (from left), a half cage encapsulated by Cy3-labeled GOx; lane 2: a half cage encapsulated by Cy5-labeled HRP; lanes 3 and 4: a full cage (dimer of two half cages) encapsulated by both GOx and HRP. The gel is visualized in three imaging channels: EB: visualization of ethidium-bromide-stained DNA structures. Cy3: visualization of Cy3-labeled GOx. Cy5: visualization of Cy5-labeled HRP[33]. (**c**) AFM imaging of GOx–HRP pairs assembled on a rectangular DNA origami tile, where the assembly of two enzymes results in the brighter spots due to the increased height compared with the surface of a DNA origami. Scale bar, 100 nm; inset magnification, 3×. (**d**) Negatively stained TEM images of DNA cages containing a GOx–HRP pairs (shown as less stained dots). Scale bar, 50 nm (ref. 33). (**e**) CCD images of representative fields of view for the smTIRF characterization of the colocalization of a Cy3-labeled enzyme and a Cy5-labeled DNA cage. Examples of Cy3–Cy5 colocalization are highlighted using a pair of rectangles[33]. (**f**) Fluorophore photobleaching trajectories with one, two and three photobleaching steps for enzyme-encapsulated DNA cages. Photobleaching steps were quantitatively analyzed by fitting the trajectories by HMM in the QUB program[33]. (**g**) Photobleaching statistics for Cy3-labeled enzymes encapsulated within a full cage and for a nonencapsulated enzyme control[33]. **a** adapted with permission from ref. 28, Nature Publishing Group. **b** and **d**–**g** reproduced with permission from ref. 33, Nature Publishing Group.

Box 3 | AGE characterization and purification ● TIMING 4–6 h

Unlike PAGE for small DNA nanostructures, AGE is used to characterize and purify large enzyme-assembled DNA nanostructures (e.g., DNA origami). Below, we describe a protocol for the characterization and purification of enzyme-assembled DNA origami structures with ~20–30% recovery yield. The B2 Mini Gel Electrophoresis System is used to perform the AGE.

Procedure

Gel preparation

1. For the preparation of 120 ml of 2% agarose gel, 2.4 g of agarose should be added to 120 ml of 1× TAE buffer in a 600-ml beaker.
2. Microwave the solution on high power for 3 min until bubbles start to appear. Gently swirl the solution to dissolve the solid. Then microwave the solution again. Repeat these steps a few more times until all of the agarose has fully dissolved in the buffer to form a clear solution.

! **CAUTION** Wear heat-insulating gloves to protect your hands from the boiling agarose solution and the hot bottle.

! **CAUTION** Avoid microwaving longer than 3 min, as it may result in the solution exploding.

3. If a substantial amount of liquid has evaporated, add water to a final volume of 120 ml.
4. Add 1 ml of 1.2 M $MgCl_2$ to the agarose solution for a final concentration of ~10 mM Mg^{2+}.
5. Add 12 µl of SYBR Safe (~ 1:10,000 dilution) to the agarose solution and swirl the solution gently until it is evenly distributed.

! **CAUTION** SYBR Safe is toxic; wear gloves.

▲ **CRITICAL STEP** Do not add staining dye directly to the very hot solution; instead, cool the solution on the lab bench for a few minutes before adding SYBR Safe.

6. Pour the gel into the casting tray and insert a gel comb. Wait for 1 h at room temperature for the gel to solidify.

Gel running

7. After the gel has solidified, assemble the casting tray in the gel box. Fill the gel box with 1× TAE buffer containing 10 mM $MgCl_2$.

▲ **CRITICAL STEP** It is recommended to incubate the gel box in an ice–water bath to prevent heat damage to gel and DNA structures during electrophoresis.

8. Remove the comb carefully. Load ~150 µl of DNA nanostructure sample into each of the gel pockets.
9. Set the voltage to 60 V and run the gel for 2–4 h, depending on the size of the structures.
10. After running the gel, the UV-visible light transilluminator is used to visualize the SYBR Safe–stained DNA nanostructures.

! **CAUTION** Wear goggles, gloves and lab coats with long sleeves to protect your skin and eyes from the harmful UV radiation.

The extraction of DNA nanostructures from the gel

11. Under UV illumination, use a razor blade to cut out the DNA band of interest, and further chop it into small pieces and transfer them to a Freeze 'N Squeeze DNA gel extraction spin column.

▲ **CRITICAL STEP** Cut out as much as possible of the DNA band of interest in order to improve the recovery yield. It is also critical to cut it into very small pieces to facilitate the elution of DNA structures from the gel.

12. Freeze the spin column samples at 20 °C for 5 min and then centrifuge them at 10,000*g* at 4 °C for 8 min. Discard the agarose debris remaining on the top of the filter cup. Recover the DNA nanostructure solution from the bottom of the tube, and store it at 4 °C for up to ~6 months.

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enzymes, organization of geometric arrangements and mimicry of cellular substrate channeling and compartmentalization.

- *Control over spacing distance between enzymes*. Multiple enzymes can be positioned on DNA nanoscaffolds with controlled distances ranging from a few nanometers (~3 nm = shortest distance) to tens of nanometers. This controlled spacing can be used to investigate and optimize mass transport of substrates between enzymes in biochemical pathways[25–27].
- *Control over stoichiometry of enzymes and cofactors*. The numbers of anchored enzymes and cofactors are controlled by the number of introduced capture sites that are displayed on the surface of DNA nanoscaffolds[28]. The relative stoichiometry between different components can be easily adjusted in order to maximize the overall catalytic efficiency of the assembled pathway.
- *Optimization of the geometric arrangement of multienzyme assembly*. Multiple enzymes can be organized with more geometric patterns than the linear pattern on 2D and 3D DNA nanostructures, such as four-way junctions[28], triangular patterns[50] and protein arrays[26].
- *Enzyme encapsulation within 3D DNA nanocages*. A series of 3D DNA–based objects such as nanocages[32,51], tetrahedrons[52] and nanoboxes[31] can be used for enzyme encapsulation. DNA nanocage–encapsulated enzymes have been reported to exhibit enhanced enzyme activity and increased stability against biological degradation, with potential applications in biocatalysis and drug delivery[33].
- *Mimicry of substrate channeling*. The direct mass transport of substrates from one enzyme to a proximal partner can greatly improve the catalytic efficiency in natural systems. The combination of the addressability of DNA nanostructures with a bioconjugation strategy makes it possible to rationally design and modify chemical properties at desired locations. For example, a NAD^+-modified ssDNA was reported to transfer hydrides between a pair of dehydrogenases on DNA nanoscaffolds, mimicking the swinging arm in the pyruvate dehydrogenase complex[28]. It is possible to further constrain the diffusion of substrates between enzymes by modifying the local pathway between them with specific properties (e.g., polarity or hydrophobicity) and substrate-binding affinity (e.g., aptamers)[53,54].

Box 4 | AFM/TEM imaging of enzyme assembly ● TIMING 1–2 h

Gel electrophoresis gives limited information about the sizes of nanostructures, without further characterization of the structural conformation and integrity. For direct observation of enzyme assembly on DNA nanostructures, two main techniques are used—AFM and negatively staining TEM. AFM is particularly useful for imaging 2D DNA nanostructures such as the rectangular DNA origami in shown in **Figure 5c**. TEM can image 3D DNA nanostructures, as well as enzyme encapsulation within the DNA nanostructures (**Fig. 5d**).

AFM imaging procedure

AFM imaging is performed using the Multimode 8 (Bruker) instrument with the ScanAsyst module.

1. Turn on the AFM system as described in the instrument setup.

! CAUTION Avoid looking directly at the laser beam with the eyes.

2. Use a double-sided tape to glue on and tear off a few layers of from the mica disk in order to get a clean and flat surface. Deposit 2 µl of an ~10 nM DNA origami sample (for small tiles, use ~200 nM) onto the center of a mica surface. Wait for 2 min and then add ~40–50 µl of 1× TAE Mg^{2+} buffer (pH 8.0). The DNA nanostructures are tightly bound to the mica surface because of the electrostatic interaction. (Optional) To enhance the adsorption of DNA nanostructures onto the mica surface, 2 µl of 100 mM $NiCl_2$ solution should be added to the DNA solution on the mica surface.

! CAUTION $NiCl_2$ is toxic and may cause respiratory sensitization; wear gloves.

▲ CRITICAL STEP For imaging enzyme assembly in solution, do not dry the sample on the mica surface, but keep it in liquid form.

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3. Assemble a ScanAsyst Fluid+ AFM tip onto the liquid cell.
4. Transfer the sample disk onto the AFM scanner and secure the liquid cell on the top of the sample disk.

▲ CRITICAL STEP The scanner stage must be set at a low position. Carefully place the tip holder onto the top of the sample solution without touching the mica surface. Avoid leakage of solution into the AFM scanner.

5. Open the control software for ScanAsyst in Fluid, and set the initial scan size to 2 µm and the Samples/Line to 256 or higher. Next, gradually reduce the peakforce set-point to decrease the force applied to the sample, in order to avoid damage to the structures due to the tip scraping.

▲ CRITICAL STEP Proteins may be torn off from the DNA nanostructures if too much force is applied during scanning. Carefully adjust the peakforce set-point to obtain clear and accurate images of assembled enzyme–DNA structures.

AFM data analysis

Most 2D DNA structures (e.g., origami tiles) are deposited on the mica surface with the protein-decorated side facing up, probably because of the strong interaction (charge or stacking) between the opposite flat side and the mica surface. Thus, AFM imaging can be used to count the assembly yield of enzymes on the DNA nanostructures. An example is shown in **Supplementary Figure 3**.

6. Open the raw image file in the NanoScope Analysis software.
7. Execute a Flatten function to correct image for tilt and bow.
8. The assembled enzymes typically result in brighter spots because of their increased height compared with the surface of the DNA structure (**Fig. 5c**).
9. The researcher can zoom in to areas of interest for detailed analysis. The Section function can be used to measure parameters such as height and distance.
10. Analyzed images should be converted to JPEG or PNG formats for future presentations.

TEM imaging procedure

TEM imaging is performed using the JEM-1400 (JEOL), and images are analyzed using the ImageJ software.

Sample preparation

11. Before depositing the sample, the EM grids should be negatively glow-discharged using an Emitech K100× machine at 45 mA for 30 s.
12. Deposit 2 µl of DNA sample solution onto a carbon-coated copper grid; let it incubate for 2 min, and then remove the solution from the grid by absorbing it with a piece of filter paper at the edge of the grid.

! CAUTION Do not fully dry the grid, but leave a thin layer of solution.

13. Add 6 µl of the staining solution (1% (wt/vol) uranyl formate) to the grid and incubate for 15 s; then remove the solution using a piece of filter paper, as described in the above step. Leave the grid exposed to air for 30 min to dry completely.
14. Store the stained grid in a grid box and label it with the sample name.

TEM imaging

15. Insert the sample into the transmission electron microscope and image at a working voltage of 80 kV.

▲ CRITICAL STEP Caged DNA origami, with enzyme encapsulated, can be observed in TEM images.

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- *Regulatory enzyme nanoreactor*. Switchable DNA nanostructures can be used to regulate the spatial interactions between enzyme pairs or enzymes and cofactors for actuating catalytic activities. Examples include DNA tweezers–actuated catalytic reaction of a dehydrogenase and a cofactor[29], and an enzyme cascade reaction[30].

Box 5 | smTIRF characterization of DNA nanocaged enzymes ● TIMING 4–6 h

In the prism-based TIRF used in this protocol, a prism is placed onto a microscope slide and an objective is contacted with the opposite face of a sample chamber. A laser beam is coupled to an object of high refractive index at an angle larger than the critical angle for creating a total internal reflection at the interface. The total internal reflection induces an evanescent wave or field that illuminates a limited specimen region (~50- to 150-nm thick) immediately adjacent to the interface, such as a contact area between a glass coverslip and a specimen. The ultralow background is achieved by confining the excitation volume to a small region of the sample near the surface. For more detailed information, please see the instrument setup. The procedure for prism-based TIRF microscopy is described below for characterizing enzyme–DNA assemblies and enzyme activities:

Procedure

1. Add a drop of water onto the 60× objective, and then mount a microfluidic PEGylated slide onto the microscope stage with the microscope slide facing up (the detailed preparation is described in PROCEDURE Steps 44–52).
2. Inject 200 µl of 0.2 mg/ml streptavidin (prepared in T50 buffer) into the flow channel, and incubate for 10 min at room temperature. Flush out the excess streptavidin using the same buffer. The channel surface is now immobilized with streptavidin for capturing the biotinylated DNA nanostructures.
3. First add a drop of water on the objective, and then load the slide onto the objective. Add a drop of immersion oil on top of the microscope slide, and place the prism in tight contact with the slide, using the prism holder.
4. Focus a low-power laser (green and/or red) onto the surface of the microscope slide by moving the objective toward the slide.

! CAUTION Avoid laser exposure to the eyes, and wear laser safety goggles.

? TROUBLESHOOTING

5. Use a syringe with a needle to inject 300 µl of imaging buffer (e.g., 1× TAE–Mg^{2+} for DNA nanostructures) through the microfluidic channel.

▲ CRITICAL STEP Use both green and red lasers to focus onto the microscope slide before adding the sample. Photobleach the slide surface, if there is any fluorescent debris, by exciting the specimen area at a high laser power for ~10–15 min until the background fluorescence has been completely removed. If there is still fluorescent debris after photobleaching, replace it with a new microscope slide.

6. Prepare biotinylated DNA nanostructures in the imaging buffer with the addition of the oxygen scavenger solution (OSS; please see Reagent Setup for its preparation). Close the shutter to prevent laser illumination. Flow a DNA–enzyme assembly solution of 30–100 pM through the channel in the dark and incubate for 1–3 min for immobilizing the sample on the streptavidin-coated surface. To optimize the immobilization density (typically ~0.06 molecules per μm^2), open the shutter quickly to image the sample every ~30 s.

? TROUBLESHOOTING

7. Flush out the unbound sample using the imaging buffer containing OSS. Image the sample at room temperature with an appropriate camera integration time. For the fluorescence colocalization and the stepwise photobleaching in **Figure 5e–g**, we used a 100-ms integration time. For characterizing enzyme turnovers, a shorter integration time of 35 ms should be used.
8. Set the laser power to ~15 W/cm^2 for the 532-nm laser and to ~40 W/cm^2 for the 638-nm laser; set the microchannel plate (MCP, an image intensifier controller) gain to ~70 for imaging fluorophore spots in the field of view. A shuttered illumination may be used to prevent the fluorophores from photobleaching.
9. Save the imaging movie with the data acquisition program (written in MATLAB). Detailed data processing and analysis have been described in the Experimental design section.

Comparison with other methods

In addition to DNA nanostructures, a few other techniques have been developed to organize and assemble multienzyme pathways[55], including genetic fusion of protein scaffolds[56,57], chemical cross-linking, liposome compartmentalization and surface coimmobilization. Genetically fused protein scaffolds have been demonstrated to assemble a series of enzymes in a cascade pathway for boosting the cellular metabolic flux by up to 77-fold[56]. The high-affinity interaction between cohesin and dockerin modules was used to assemble multienzyme complexes in which dockerin-fused enzymes were captured on a mini-scaffold containing the corresponding cohesins[57]. The genetically fused protein scaffolds can be applied to *in vivo* systems and may be produced in large scale by bacteria fermentation, but they lack the ability to offer more precise control over geometric arrangements in 2D and 3D patterns than the simple linear pattern. Chemical cross-linking of enzyme aggregates has been reported to enhance enzyme stability in biocatalysis applications[58]. Surface coimmobilization of multiple enzymes has also been used to optimize mass transport of substrates between enzymes, and to increase the enzyme density for promoting catalysis[59]. However, the performance of immobilized enzyme systems is often limited by inherent difficulties associated with surfaces, such as protein denaturation due to nonspecific absorption[60,61], the inability to control enzyme orientation and conformation on surfaces[59,61] and variations in the spatial distances between enzymes, as well as the enzyme-to-surface distance[62]. Compartmentalization of enzymes is realized using several methods, such as virus-like protein particles[63,64], liposomes[65], polymersomes[66] and chemical cross-linking[67], which allow the large-scale production of artificial enzyme particles. Severe obstacles prevent these enzyme particles from having a broader application, including low encapsulation yield of large proteins because of steric hindrance[68], insufficient access of substrates to encapsulated enzymes[64], aggregation of vesicle shells[64,69] and limited control over the spatial arrangement (e.g., distance) of proteins within the compartments[63,70]. Recent developments in RNA nanotechnology have also been exploited to organize multienzyme systems *in vivo* with a level of spatial organization similar to that of DNA nanostructures[71]. The *in vivo* assembly of nucleic acid nanoscaffolds offers a new approach for synthetic

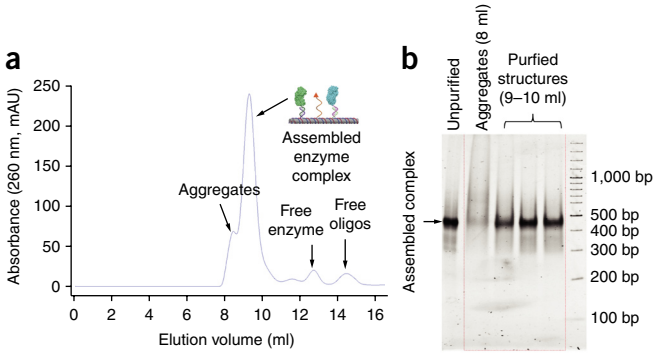

Figure 6 | Purification and structural characterization of enzyme-assembled DNA nanostructures. (**a**) Size-exclusion FPLC purification of a fully assembled G6PDH-NAD$^+$-MDH structure to get rid of excess enzymes and free DNA oligos. Running buffer: 100 mM HEPES (pH 8). (**b**) 3% (vol/vol) native PAGE characterization of the structures collected in size-exclusion FPLC: the 8-ml fraction contains aggregated structures with smear bands. The 9- to 10-ml fraction comprises the fully assembled enzyme structures. The unpurified structure is also shown in the left lane as a control, which contains incomplete assemblies, aggregations and free proteins.

biology to engineer and regulate novel cellular metabolic pathways, such as the promotion of bacterial hydrogen production[72]. However, the relative instability of RNA molecules makes it more difficult to translate these nanostructures to noncellular and cellular environments.

Limitations

Although self-assembled DNA nanostructures have demonstrated great potential for organizing multienzyme complexes on the nanoscale, there are limitations of current techniques that require future improvements for broader applications. First, DNA nanostructures organize multienzyme complexes with nanometer precision (3–5 nm resolution), not Ångstrom precision. Thus, it is difficult to manipulate molecular assemblies at an atomic level, as would be required for the arrangement of amino acids in the enzyme active site, as an example. Second, the current protocol uses a non-site-specific chemistry (e.g., lysine-based conjugation) to conjugate DNA to an enzyme. Due to the presence of multiple lysines on most protein surfaces, lysine-based conjugation results in heterogeneous attachment sites for DNA. This heterogeneity may affect the activities of DNA-conjugated enzymes because of their varied orientations on a DNA nanostructure. Recent progress in site-specific protein conjugation chemistry[37,73,74] can be applied to coupling DNA molecules more precisely to a protein surface. Using site-specific DNA–enzyme conjugation, the orientation of an enzyme can be better controlled by using two or three capture strands to orient the enzyme on the DNA nanoscaffold. Third, the current protocol was demonstrated successfully for assembling and characterizing nanostructure complexes consisting of only a few pairs of enzymes[25,28,29,33,49]. One should realize that the overall assembly yield may be reduced when increasing the size and number of components in the assembled structures (e.g., for protein arrays). This overall assembly yield can be estimated based on a calculation similar to that used for peptide or nucleotide synthesis: $Y_{overall} \sim (p)^n$, where p is the average assembly yield for individual enzymes and n is the total number of assembled enzymes. The assembly yield also decreases for densely packed enzyme complexes because of the steric crowding between enzymes. For example, as shown in **Figure 1e–p**, the assembly yield of a GOx–HRP pair on a DNA origami tile increases from ~50% for 10-nm spacing to >90% for 45-nm spacing[25]. The stability of enzyme-assembled DNA nanostructures is another concern because of the possible disassembly of DNA nanostructures at low Mg^{2+} concentrations[75]. In our tests, the structures of assembled enzyme complexes were stable over a week in a buffer (1× TAE or 100 mM HEPES) containing 12.5 mM Mg^{2+} when stored at 4 °C (refs. 25,28,29,33). The assembled complexes also stay structurally intact and maintain integrity after enzyme reactions[25,28,33].

Single-molecule fluorescence characterization of enzyme kinetics is limited to enzyme systems that form stable fluorescent products such as fluorescein and resorufin. There are limited choices of fluorescent analogs for enzyme substrates on the market. The nonspecific conversion of the substrate into fluorescent product may give a false-positive signal—e.g., through the photo-oxidation of Amplex-Red into resorufin[76]. The frame rate of the charge-coupled device (CCD) camera may limit the time resolution of the measurement; e.g., the highest CCD frame rate in our current smTIRF setup is ~100 Hz (~10 ms per frame). A higher frame rate can be achieved by using a smaller chip area, but doing so will also reduce the number of molecules that are recorded in the field of view. Single-molecule sensitivity also requires that the sample have very low autofluorescence at the excitation wavelength. Some additional application limitations to be tackled in the future include the relatively low concentration achievable, the small production scale of DNA-organized enzyme complexes and the high cost of synthetic oligonucleotides[77]. Typical concentrations for producing large DNA origami nanostructures are in the low-nanometer range[25], whereas the concentration of small DNA tiles can be in the micromolar range[28,29]. This production scale is several orders of magnitude lower than standard enzyme production in bacteria. The recent development of scalable amplification of DNA nanostructures[77–80] may provide a solution and enable an increase in the production scale of DNA-organized multienzyme complexes in the future.

Experimental design

DNA labeling of enzyme. Before SPDP labeling, the enzyme solution must be exchanged into a buffer solution without primary amines, such as HEPES or phosphate buffer. The presence of primary amines in the buffer (e.g., Tris) can react with SPDP and decrease the labeling efficiency. The SPDP labeling reaction is also highly sensitive to the pH values of the reaction solution. For a neutral or acidic pH, the primary amine on the side chain of the lysine residue is protonated because of its high pK_a (acid dissociation constant; ~10.2), and thus it reacts slowly with the NHS group. The SPDP labeling reaction is kept at pH ~8.5 for deprotonating the primary amines. After the reaction, it is important to determine the labeling ratio of SPDP molecules to enzyme. T-CEP (tris(2-carboxyethyl)phosphine) is added to the solution of SPDP-modified enzymes for reducing the disulfide bond to release pyridine-2-thione, resulting in an increased absorbance at 343 nm (extinction coefficient: 8,080per M per cm). The labeling ratio of SPDP molecules can be estimated using equation 1 as shown below:

$$\text{SPDP labeling ratio} = \frac{\frac{\Delta 343}{3080}}{[\text{Enzyme}]} \quad (1)$$

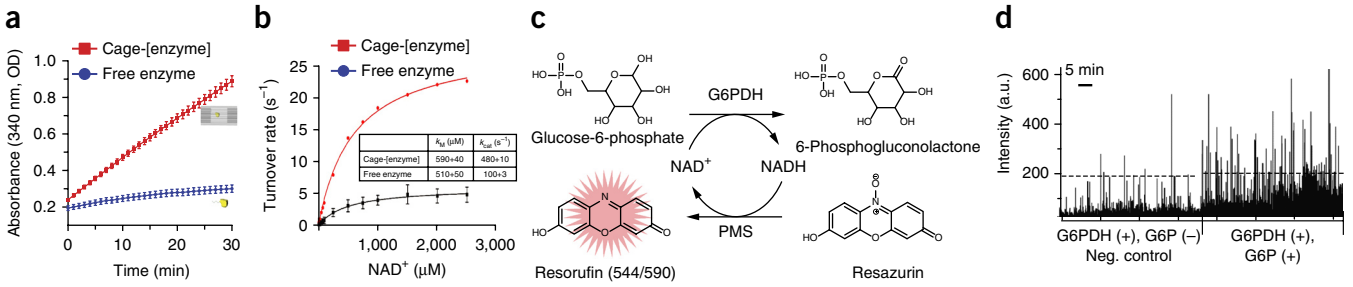

Figure 7 | Activity characterization of the enzyme-assembled DNA nanostructures. (**a**) Plots of product [NADH] versus time for a cage-[G6PDH] complex and a free enzyme. The production of NADH is directly monitored by the increased absorbance at 340 nm. Error bars represent the standard deviation of three replicates ($N = 3$). (**b**) The Michaelis–Menten kinetical analysis of a cage-[G6PDH] complex and a free G6PDH enzyme, performed by titrating the enzyme activities depending on the increased concentration of NAD^+. Error bars represent the standard deviation of three replicates ($N = 3$). (**c**) A PMS/resazurin-coupled fluorescence assay is used to characterize the activity of G6PDH. NAD^+ is first reduced to NADH by G6PDH, followed by PMS-catalyzed electron transfer from NADH to resazurin, producing a strongly fluorescent resorufin, which has an excitation/emission maximum at 544/590 nm. (**d**) smTIRF characterization of the real-time traces of fluorescence spikes (resorufin production) for G6PDH with and without the addition of G6P substrate. Statistics for spike frequency and fraction of active molecules are used to characterize the overall enzyme activity. smTIRF experiments are carried out at room temperature in a 1× TBS buffer, pH 7.5, in the presence of 1 mM Mg^{2+} and 10% (wt/vol) PEG 8000. **d** adapted with permission from ref. 33, Nature Publishing Group.

As shown in **Figure 8**, the activities of SPDP-labeled enzymes are tested depending on the labeling ratio of SPDP molecules to enzyme. Among the five tested enzymes, GOx is affected least upon SPDP labeling, maintaining ~80% activity of unmodified enzyme with even six labeled SPDP molecules per enzyme on average. However, the activities of other enzymes are more seriously reduced when the number of labeled SPDP molecules per protein is more than 2 or 3. These decreased activities may be due to the chemical neutralization of surface lysine residues (lysine is basic) by the SPDP labeling, which causes enzymes' isoelectric point (PI) values to be more acidic, or due to the partial structural denaturation that occurs during the chemical conjugation process. To maintain the activity of enzymes, we generally label ~1–2 SPDP molecules per enzyme on average. Next, SPDP-modified enzyme is reacted with a thiol-modified oligonucleotide through a disulfide bond exchange of the activated pyridyldithiol group.

The concentration of DNA-conjugated enzyme is estimated using the 260-nm and 280-nm absorbance as shown below:

$$A_{260} = \varepsilon_{260,enzyme} * Conc._{enzyme} + \varepsilon_{260,DNA} * Conc._{DNA} \quad (2)$$

$$A_{280} = \varepsilon_{280,enzyme} * Conc._{enzyme} + \varepsilon_{280,DNA} * Conc._{DNA} \quad (3)$$

$$Ratio\left(\frac{DNA}{enzyme}\right) = \frac{Conc.DNA}{Conc.enzyme} \quad (4)$$

The theoretical extinction coefficients for oligonucleotides are obtained from the Integrated DNA Technology (IDT) Biophysics website (http://biophysics.idtdna.com/). For enzymes containing chromophores, such as HRP with heme cofactor ($\varepsilon_{405\,nm}$ ~100,000 per M per cm), the enzyme concentration can be determined by the absorbance of chromophores.

Oligonucleotide design and purification. DNA nanostructures are designed using open-access software: Tiamat[19], cadnano[17] and NanoEngineer (Nanorex)[28]. The oligo analyzer program provided by IDT is used to check the thermal parameters (e.g., melting temperature) of the oligonucleotides, and to avoid of secondary structures. The accurate assembly of DNA nanostructures depends highly on the exact matching of the sequences and stoichiometry of the oligonucleotides, even though the DNA origami[6] and DNA bricks[7] can form well-defined nanostructures with unpurified strands. Certain key strands, such as capture strands, are required to be purified for achieving the high-yield assembly of enzymes onto DNA nanostructures. For smaller DNA tiles (that do not require the use of a scaffold), it is crucial to check the integrity, purity and concentration of all the component strands. Most oligonucleotides purchased from commercial companies (e.g., IDTs) are crude products, containing fragments of truncated or deleted sequences. Thus, denaturing PAGE is used to purify the oligonucleotides before the structural assembly. For a typical purification process, denaturing PAGE gels (6–8% (vol/vol), containing 8.3 M urea) are prepared at room temperature (~25 °C). The crude DNA strands are loaded into the gel wells and are run for 1–1.5 h at 35 °C at a constant current of 90 mA. Under UV illumination, the migrated bands corresponding to the correct strand length are first cut out from the gel, and then they are chopped into small pieces and incubated with the elution buffer overnight at room temperature. The DNA strands are extracted from the gel pieces by centrifugation using a Costar Spin X filter at 9,400*g* at room temperature for 5 min. Finally, DNA molecules are ethanol-precipitated and are dried using a Vacufuge at 30 °C for ~3–6 h. The solid DNA is dissolved in deionized water (dI H_2O), and the concentrations of individual purified strands are determined by UV absorbance at 260 nm using the extinction coefficients provided by the manufacturer.

smTIRF colocalization. The microscope setup is described in the Equipment Setup section. The smTIRF procedure is described in **Box 5**. Single-molecule fluorescence trajectories recorded from the experiments are analyzed using custom-written IDL (Interactive Data Language) and MATLAB scripts, as described previously[81]; these scripts are also available in the **Supplementary Software**.

All single-molecule measurements are performed at room temperature using a smTIRF microscope on PEGylated fused silica microscope slides. The surface of the slides is functionalized by PEGlyated biotin. Biotin–streptavidin–biotin sandwich immobilization is used to capture the biotinylated DNA–enzyme complexes on the slides[28,33]: first, streptavidin is added to the surface, then, the biotinylated DNA–enzyme complexes are captured. For the fluorescence colocalization of enzyme-encapsulated

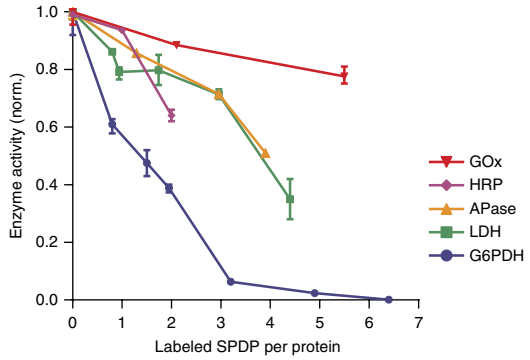

Figure 8 | Enzyme activities *vs* labeled number of SPDP molecules. Assay conditions are described as follows: 1 nM GOx is measured with the addition of 1 mM glucose, 1 mM ABTS and 10 nM wild-type HRP in 1× TBS buffer (pH 7.5), with monitoring of the increased absorbance at 420 nm. 1 nM HRP is measured with the addition of 1 mM glucose, 1 mM ABTS and 10 nM wild-type GOx in 1× TBS buffer (pH 7.5), with monitoring of the increased absorbance at 420 nm. The activity of 20 nM APase is evaluated by adding 200 μM PNPP in 1× TBS buffer (pH 7.5), with monitoring of the increased absorbance at 405 nm. 2 nM G6PDH is evaluated by adding 1 mM G6P and 1 mM NAD+ in 1× TBS buffer (pH 7.5), with monitoring of the increased absorbance at 340 nm. 1 nM LDH is evaluated by adding 1 mM pyruvate and 1 mM NADH (LDH substrates) in 1×TBS buffer (pH 7.5), with monitoring of the decreased absorbance at 340 nm. Error bars are calculated from the standard deviation of three replicates ($N = 3$). All above results indicate that activity of enzymes may be significantly damaged with an SPDP label ratio >2.

DNA nanocages (**Fig. 5e**), a DNA-conjugated enzyme is labeled with a Cy3 molecule at the 3′ end of the oligonucleotide (e.g., enzyme-5′-TTTTTCCCTCCCTCC-3′-Cy3), and a DNA nanocage is labeled with a Cy5 molecule (ref. 33). The DNA nanocage is also labeled with three biotin molecules for the immobilization on the streptavidin-coated slides. To differentiate between the fluorescence signals from the sample and the background noise, a threshold of 150 counts per frame is used to pick up the sample particles (typically 500–1,000 counts per frame). The average encapsulation yield of an enzyme within a DNA nanocage is estimated by comparing the number of molecules containing the colocalized Cy3/Cy5 signals ($N_{coloc.}$, representing both enzymes and cages) with the number of molecules exhibiting a Cy5 signal (N_{cy5}, representing DNA cages):

$$\text{Encapsulation yield} \sim \frac{\sim r_{cy3,label} * N_{coloc.}}{N_{Cys}} \quad (5)$$

where $r_{cy3,label}$ is the average labeling ratio of Cy3 to DNA-conjugated enzymes. This ratio can be quantified using a UV-visible light spectrometer. It should be noted that if the measured Cy3 labeling ratio is >1, $r_{cy3,label}$ is still defined as 1 in equation (5). The cage is only evaluated to check whether it contains Cy3-labeled enzymes. Even if there is more than one Cy3 observed in a cage, it is counted as 1 for estimating the encapsulation yield, under the assumption that a multiply labeled enzyme is encapsulated. By contrast, DNA nanostructures (e.g., DNA cages) are assembled with five Cy5-labeled strands, which makes it very unlikely that there will be a cage without any Cy5. The DNA nanostructures without labeled Cy5 would not be observed at all and would not be counted. Thus, the Cy5 labeling ratio is not considered in equation (5). To improve the dye labeling yield, dye-labeled DNA molecules can be purified by HPLC to remove unlabeled DNA molecules. The dye-labeled DNA molecules are then conjugated with enzymes, followed by FPLC purification to collect enzymes labeled with one or two DNA molecules. In this way, at least one dye is coupled to each enzyme. More examples of smTIRF colocalization of the enzyme-encapsulated cages are shown in **Table 5**.

TABLE 5 | Examples of the estimation of the encapsulation efficiency of six different enzymes within DNA nanocages by fluorescence colocalization.

	N	N_{coloc}	N_{cy5}	N_{coloc}/N_{cage}
HRP	176	156	165	0.94
GOx	205	197	201	0.98
G6PDH	218	209	214	0.98
LDH	1,229	826	1,008	0.82
MDH	363	335	348	0.96
β-Gal	284	115	179	0.64

Enzyme encapsulation is calculated by taking the ratio of the number of colocalized molecules (i.e., both Cy3-labeled enzyme and the Cy5-labeled cage) to the total number of Cy5-labeled cages. N is the number of particles analyzed, N_{coloc} is the number of particles containing both Cy3 and Cy5 fluorophores and N_{cage} is the number of particles showing evidence of Cy5-labeled DNA cages. This table is adapted with permission from ref. 33, Nature Publishing Group.

SMPB to determine the number of encapsulated enzymes per DNA cage. Photobleaching is the photochemical process of destroying a fluorophore structure to make it permanently unable to fluoresce; it is caused by the photo-oxidation of chemical bonds, bond cleavage or nonspecific reactions between the fluorophore and the surrounding molecules. Because there are almost no synchronized photobleaching processes between two individual fluorophores, the measurement of photobleaching steps can be used to count the number of fluorophores in the analyzed sample. To count the steps, the DNA samples are illuminated with high power from a 532-nm laser (~15 W/cm^2) until all of the Cy3 fluorophores in the field of view are photobleached. The recorded fluorescence signals are first idealized in the QuB program (http://www.qub.buffalo.edu) using a finite-state (e.g., six-state) model. The histogram for the photobleaching steps is then generated using a custom-written MATLAB script. Using an example image, a detailed step-by-step guide to the photobleaching analysis is described in the **Supplementary Methods**. The intensity traces representing one, two and three photobleaching steps are shown in **Figure 5f**. The observed steps of photobleaching are used to count the number of Cy3 molecules per DNA nanocage (N_{Cy3_cage}). For the free enzyme control, a biotinylated capture oligonucleotide (5′-biotin-TTTTTGGAGGGAGGG) is first immobilized on the streptavidin-coated biotin-PEG slide, followed by hybridization with a DNA-conjugated Cy3-labeled enzyme. As shown in **Figure 5g**, the number of enzymes encapsulated within a cage ($N_{Enz.\_encap.}$) is estimated by normalizing the number of Cy3 fluorophores per DNA nanocage (N_{Cy3_cage}) to the average number of Cy3 labels per free enzyme ($N_{Cy3\_enz.}$) using equation (6).

$$N_{Enz.\_encap.} \sim \frac{N_{Cy3\_cage}}{N_{Cy3\_enz.}} \quad (6)$$

It should be noted that the labeling efficiency of enzymes with a fluorophore may affect the quantification of enzymes per cage in equation (6). Therefore, for a more reliable quantification, we take the average ratio of the number of photobleaching steps for DNA nanocaged enzymes to that of free enzymes to estimate the number of enzyme copies per cage. To improve the dye-labeling yield, dye-labeled DNA molecules can be purified by HPLC to remove unlabeled DNA molecules. The dye-labeled DNA molecules are then conjugated with enzymes, followed by FPLC purification to collect enzymes labeled with one or two DNA molecules. In this way, at least one dye is coupled to each enzyme. To improve the accuracy of quantification, we also use TEM to directly visualize the encapsulation of the two enzymes, as shown in **Figure 5d**, in combination with single-molecule photobleaching experiments.

Single-molecule enzyme activity analysis. The streptavidin-modified PEGylated slides are first incubated for ~2 min with neutravidin-coated fluorescent beads (Invitrogen, 0.04-µm diameter; excitation/emission: 550/605 nm) at 10^6-fold dilution, and excess beads are flushed out with 1× T50 (pH 8) buffer. Because these fluorescent beads (~5–8 per field of view) are highly photostable against laser illumination, they are used as fiducial markers to correct the image drifts from the microscope stage and slides during the measurement (**Fig. 9a**). Then, the enzyme-encapsulated DNA cages are loaded into the microfluidic channel and are recorded for the positions of the signals of Cy3-labeled DNA–caged enzyme. The fluorescence background in the field of view is completely removed by photobleaching with high-power laser illumination (~15 W/cm^2 for 532 nm, typically for ~10–15 min) before the loading of the enzyme substrates (the fiducial beads are highly photostable and cannot be bleached). After the measurement, the recorded movies are coordinated using the fiducial markers (visible throughout all sequential movies) in a custom-written MATLAB script. This approach allows us to infer the locations (x and y coordinates) of all individual Cy3-labeled enzymes and nanocages in the field of view during the measurement, in order to monitor catalytic turnovers (fluorescence spikes from resorufin formation) of an enzyme at a specific location.

To monitor enzyme activity of G6PDH, 300 µl of a substrate solution (1 mM G6P, 1 mM NAD^+, 50 nM resazurin and 12.5 µM PMS in 1× Tris-buffered saline (TBS)-Mg^{2+}-PEG buffer (pH 7.5, 1 mM Mg^{2+} and 10% (wt/vol) PEG8000)) is injected into the flow channel. After injecting the substrate solution, movies are recorded for ~5 min (9,091 frames) at a 35-ms frame exposure time. The activities of individual enzymes are confirmed by comparing fluorescence spikes from the same field of view with and without the addition of substrate (e.g., G6P). To determine the threshold for differentiating signals from background noise, an intensity histogram of the combined data of all traces from a movie is plotted in Origin. The histogram is fitted with a single-peak Gaussian function to determine the mean intensity and standard deviation (see **Supplementary Methods** for a step-by-step guide). In our experiment, the threshold value is defined as 'mean + 8 × s.d.' to remove random spike-like events in the control experiments. Because one or two random fluorescence spikes above the threshold are often observed in control experiments without the addition of substrate solution, only molecules exhibiting four or more spikes are counted as active molecules.

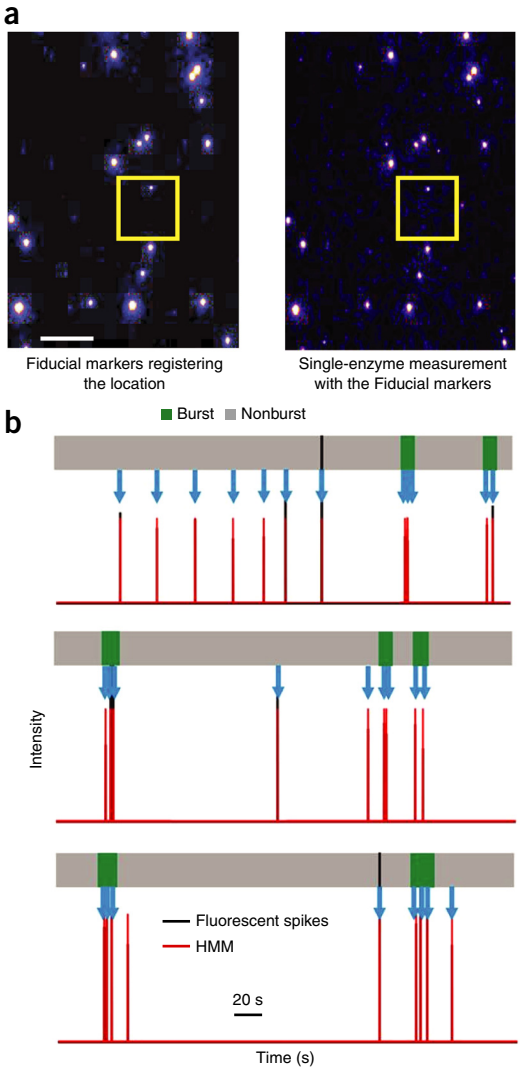

Figure 9 | Single-enzyme activity data collection and analysis. (**a**) TIRF microscope snapshots of the recorded image before and after the injection of the enzyme substrate (G6P). The addition of G6P results in increased fluorescence bursts due to the formation of resorufin—for example, in the boxed regions. Fluorescent beads (very bright spots present in both images) are used as fiducial markers to correct the drifts during the measurement. Scale bar, 10 µm. (**b**) Representative intensity–time traces (black) of full-cage enzyme after background correction and HMM idealization to a two-state model (red). The fluorescence–time traces of the enzyme reaction on the microscope slide were recorded at 35-ms time resolution over ~5 min. **a** and **b** adapted with permission from ref. 33, Nature Publishing Group.

The burst analysis is then performed only for active molecules using a modified rank surprise (RS) method[82,83] (see **Supplementary Methods** for s detailed analysis procedure). The interspike intervals (ISIs) are determined by calculating the time between individual fluorescence spikes for each molecule (**Fig. 9b**). The RS method allows the determination of the start and end points of bursts after collecting ISIs for all molecules. In this analysis, only the intensity spikes with an ISI of ≤5 s are considered part of a burst; any other intensity spikes are counted as nonbursts. In single-molecule measurement of enzyme activity, we observed that the spike frequency (which reflects individual enzyme turnovers) varies between encapsulated enzymes, which might be attributed

to the heterogeneity introduced by lysine-based DNA–enzyme conjugation. In our further analysis, we compared the overall average spike frequency of all DNA cage–encapsulated enzymes with the same DNA-conjugated, but nonencapsulated, enzymes. The activity enhancement was calculated based on the relative turnover frequencies of encapsulated and nonencapsulated enzymes.

MATERIALS

REAGENTS

! CAUTION All reagents are potentially dangerous. They should be handled only by specially trained personnel.

- Trizma base, ≥99.9% (wt/wt) (titration), crystalline (Sigma-Aldrich, cat. no. T1503)
- Acetic acid, ≥99.7% (Sigma-Aldrich, cat. no. 695092)
- EDTA, 99.4–100.6%, powder (American Chemical Society (ACS) reagent; Sigma-Aldrich, cat. no. E9884)
- Urea (Sigma-Aldrich, cat. no. U6504)
- Formamide, ≥99.5% (ACS reagent; Sigma-Aldrich, cat. no. 221198)
- Xylene cyanol FF, dye content 75% (Sigma-Aldrich, cat. no. 335940)
- Bromophenol blue (Sigma-Aldrich, cat. no. B0126)
- Glycerol, ≥99.5% (Sigma-Aldrich, cat. no. G9012)
- Ammonium acetate, ≥98%, for molecular biology (Sigma-Aldrich, cat. no. A1542)
- Magnesium acetate tetrahydrate $(CH_3COO)_2Mg{\cdot}4H_2O$, ≥99%, for molecular biology, (Sigma-Aldrich, cat. no. M5661)
- Magnesium chloride hexahydrate ($MgCl_2$; Fisher Scientific, cat. no. M35-500)
- Sodium hydroxide (NaOH) ≥98%, pellets (anhydrous), reagent grade (Sigma-Aldrich, cat. no. S5881)
- Boric acid, ≥99.5%, for electrophoresis (Sigma-Aldrich, cat. no. B7901)
- 40% Acrylamide stock solution (19:1 acrylamide:bis-acrylamide; Bio-Rad, cat. no. 1610154) **! CAUTION** It is highly toxic on skin contact, and it is known to be a potent neurotoxin. Handle the compound within an organic hood. Store it at 4 °C for up to 12 months. **▲ CRITICAL** It is important to order bis-acrylamide with a 19:1 ratio for correct polymerization.
- Tetramethylethylendiamine (TEMED; Merck, cat. no. 1.10732.0100-100 ml) **! CAUTION** TEMED is toxic, and it can cause skin and eye irritation.
- Hydrochloric acid solution (HCl) 1 N (certified; Fisher Scientific, cat. no. SA48)
- Surfactant P20 (P20, GE Healthcare, cat. no. BR100054)
- Ammonium persulfate (APS; Sigma-Aldrich, cat. no. A3578) **! CAUTION** APS is toxic if swallowed. Prepare it in 0.3-ml aliquots of 10% (wt/wt) water solution and store the aliquots at −20 °C for up to 2 months.
- Nickel(II) chloride hexahydrate ($NiCl_2{\cdot}6H_2O$; Sigma-Aldrich, cat. no. 223387)
- Sodium chloride (NaCl), ≥99.5% (Sigma-Aldrich, BioXtra, cat. no. S7653)
- Sodium phosphate monobasic monohydrate ($NaH_2PO_4{\cdot}H_2O$), ≥98% (ACS reagent; Sigma-Aldrich, cat. no. S9638)
- Sodium phosphate dibasic heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), ≥98% (ACS reagent; Sigma-Aldrich, cat. no. S9390)
- PBS, (pH 7.4, powder (Sigma-Aldrich, cat. no. P5368)
- Triethylammonium acetate (TEAA), 1 M, pH 7.0, solution (VWR, cat. no. 80108-322)
- HEPES sodium salt ($C_8H_{17}N_2NaO_4S$), ≥99.5% (Sigma-Aldrich, cat. no. H7006)
- SYBR Green I Nucleic Acid Gel Stain, 10,000× in DMSO (Thermo Fisher Scientific, cat. no. S-7585) **! CAUTION** It is toxic by ingestion and if inhaled as dust. Wear protective clothing and use it in a fume hood.
- SYBR Gold Nucleic Acid Gel Stain, 10,000× in DMSO (Thermo Fisher Scientific, cat. no. S-11494) **! CAUTION** It is toxic by ingestion and if inhaled as dust. Wear protective clothing and use it in a fume hood.
- Agarose (Thermo Fisher Scientific, cat. no. 16500-100 g)
- Uranyl formate (Electron Microscopy Sciences, cat. no. 22450-1 g) **! CAUTION** It is toxic by ingestion and if inhaled as dust. Wear protective clothing and use it in a fume hood.
- Deionized water (dI H_2O; 18.2 MΩ · cm at 25 °C)
- Glucose-6-phosphate dehydrogenase (G6PDH), from *Leuconostoc mesenteroides* lyophilized powder, 550–1,100 units per mg protein (biuret; Sigma-Aldrich, cat. no. G8529)
- L-Lactic dehydrogenase, from rabbit muscle, 800–1,200 units per mg protein (Sigma-Aldrich, cat. no. L2500)
- Alkaline phosphatase (Apase), from bovine intestinal mucosa (Sigma-Aldrich, cat. no. P5521)
- PNPP (4-nitrophenyl phosphate; Sigma-Aldrich, cat. no. N9389-50TAB)
- Glucose oxidase (Gox), from *Aspergillus Niger* (Sigma-Aldrich, cat. no. G7141)
- Horseradish peroxidase (HRP), peroxidase from horseradish, Type VI, essentially salt-free, lyophilized powder, 250–330 units per mg solid (Sigma-Aldrich, cat. no. P8375)
- Tris-buffered saline (TBS), pH 8.0, powder (Sigma-Aldrich, cat. no. T6664) or 10× buffer solution (Fisher Scientific, cat. no. BP24711)
- DMSO for molecular biology (Sigma-Aldrich, cat. no. D8418)
- T-CEP (tris-(2-carboxyethyl)-phosphine hydrochloride), ≥98%, powder (Sigma-Aldrich, cat. no. C4706)
- ABTS (2,2′-azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt), ≥98% (Sigma-Aldrich, cat. no. A1888)
- SPDP (*N*-succinimidyl 3-(2-pyridyldithio)-propionate), premium grade (Pierce, cat. no. PG82087)
- DSS (disuccinimidyl suberate; Sigma-Aldrich, cat. no. S1885-1G)
- 6AE-NAD^+ (BioLog, cat. no. N 013)
- DEAE-Sepharose resin (Sigma-Aldrich, cat. no. DFF100) **▲ CRITICAL** This resin has very high efficiency (>90%) for capturing linear DNA molecules via charge adsorption.
- *N*,*N*-Dimethylformamide (DMF), anhydrous (Sigma-Aldrich, cat. no. 227056-100ML)
- *N*,*N*-Diisopropylethylamine (DIPEA; Sigma-Aldrich, cat. no. 387649-100ML)
- Amplex UltraRed Reagent (Thermo Fisher Scientific, cat. no. A36006)
- Potassium hydroxide pellets (Fisher, cat. no. P250-1)
- Ammonium hydroxide, 28–30 wt% solution of NH_3 in water (Fisher, cat. no. 29130)
- Hydrogen peroxide, 35 wt% solution in water (Fisher, cat. no. AC20246-0010)
- Resazurin sodium salt (Fisher, cat. no. 50-700-7914)
- Phenazine methosulfate (Fisher, cat. no. AC130160010)
- D(+)-Glucose-6-phosphate sodium salt (G6P; Fisher, cat. no. AC446980010)
- Biotin–PEG–succinimidyl valeric acid (Biotin-PEG-SVA), MW 5,000 (Laysan Bio, cat. no. Biotin-PEG-SVA-5000-500 mg)
- mPEG-succinimidyl valerate, MW 5,000 (Laysan Bio, cat. no. MPEG-SVA-5000-1g)
- Disulfosuccinimidyl tartrate (Soltec Ventures, cat. no. CL107)
- Streptavidin (Invitrogen, cat. no. S-888)
- (3-Aminopropyl)triethoxysilane (APTES; Fisher, cat. no. BP 179-25)
- Biotinylated BSA (ImmunoPure, Thermo Fisher, cat. no. 29130)
- Protocatechuate 3, 4-dioxygenase (PCD), from *Pseudomonas* sp. (Sigma-Aldrich, cat. no. P8279-25UN)
- Two-component epoxy adhesive, 3.5 g, Hardman double/bubble (Hardman, cat. no. 4001)
- Protocatechuic acid (PCA; Sigma-Aldrich, cat. no. 37580-100G-F)
- Methanol, HPLC grade (Fisher, cat. no. A452-4)
- Acetone, HPLC grade (Fisher, cat. no. A949-4)
- Ethanol, 200 proof (Koptec, cat. no. V1001)
- Alconox (Fisher, cat. no. 50821294)
- Trolox (Fisher, cat. no. 218940050)
- 3-Hydroxypicolinic acid (3HPA; Sigma-Aldrich, cat. no. 56197)
- Disulfosuccinimidyltartrate (DST; Soltec Ventures, cat. no. CL107)
- Immersion oil, low-fluorescence (Olympus, cat. no. Z-81225)
- FluoSpheres (NeutrAvidin-labeled microspheres; Invitrogen, cat. no. F-8770)
- Glacial acetic acid (Fisher, cat. no. BP2401-212)
- PEG8000 (Promega, cat. no. V3011)
- M13mp18 single-stranded DNA (Affymetrix, cat. no. 71706)
- Crude oligonucleotides (25–100 nmole) **▲ CRITICAL** Oligonucleotides can be ordered from IDT. The oligonucleotides are further purified using denaturing PAGE, as described in the Experimental design. Oligonucleotides with terminus (5′ or 3′)-modified amines (Amino Modifier C6) or thiols (Thiol Modifier C6 S-S) should be ordered at the 1-μmole scale from IDT.
- Triton X-100, laboratory grade (Sigma-Aldrich, cat. no. X100-100ML)
- Sodium bicarbonate (Sigma-Aldrich, cat. no. S5761-500G)

TABLE 6 | The preparation of 35 ml of 5–20% denaturing PAGE gel, with suggested ranges of separation.

Gel percentage	5%	6%	8%	10%	12%	14%	20%
20% PAGE solution	8.75	10.5	14	17.5	21	24.5	35
0% PAGE solution	26.25	24.5	21	17.5	14	10.5	0
Separation range (nt)	70–300	45–70	35–45	25–35	<25		
Bromophenol Blue	35	26	19	12	8		
Xylene Cyanol	130	106	76	55	26		

Bromophenol blue is used as a high-mobility marker dye, and xylene cyanol is used as a low-mobility marker dye.

EQUIPMENT

- Water-circulating bath (VWR, model no. 1160S, cat. no. 13721-082)
- Vertical electrophoresis system (GE Healthcare Life Sciences, model no. SE 600 Ruby, cat. no. 80-6479-57)
- Electrophoresis power supply (Fisher Scientific, cat. no. FB1000)
- MWCO filters, 30 kDa (EMD Millipore, Amicon Ultra-4 Centrifugal Filters, cat. no. UFC803096)
- Standard heat block (VWR, cat. no. 13259-030)
- Agarose gel electrophoresis system, Owl EasyCast B2 Mini Gel Electrophoresis System (Thermo Fisher Scientific, cat. no. 09528110B)
- Freeze ′N Squeeze spin columns (Bio-Rad, cat. no. 732-6165)
- SigmaPrep spin column (Sigma-Aldrich, cat. no. SC1000-1KT)
- Refrigerated centrifuge, swing-bucket rotor, 15-amp version (Eppendorf, model no. 5804R)
- Refrigerated centrifuge, fixed-angle rotor (Eppendorf, model no. 5424 R)
- Hand-held UV lamp, 254/365-nm (UVP, model no. UVGL-58)
- Vacufuge (Eppendorf Vacufuge plus, model no. 5305)
- Thermocycler (Eppendorf Mastercycler Pro with Vapo. Protect Technology, model no. 6321)
- Gel imager (Bio-Rad, Molecular Imager Gel Doc XR+ System, cat. no. 1708195)
- Typhoon Trio plus variable mode imager, equipped with excitations at 532, 633 and 488 nm, and emissions at 520, 580, 610 and 670 nm (GE Healthcare, model no. 63-0055-86)
- Freezer at −20 °C (Fisher Scientific Isotemp General-Purpose Freezer, cat. no. 13986148)
- Freezer at −80 °C (VWR Signature Ultra-Low Temperature Upright Freezer, −86 to −50 °C, model no. 5604)
- Lyophilizer (Labconco FreeZone 2.5plus Freezer Dry System, cat. no. 7420020)
- Vortex mixer (Fisher Scientific Analog Vortex Mixer, cat. no. 02215365)
- NanoDrop 2000 (Thermo Fisher Scientific, S06497)
- HPLC system (Agilent Technologies, 1200 series, equipped with G1379B degasser, G1312A binary pump, G1329A autosampler, G1316A thermostatted column, G1321A fluorescence detector, G1315C DAD SL and G1364B preparative scale fraction, and OpenLAB Control Panel Software (version A.01.05))
- HPLC column: Clarity 3μ Oligo-RP column, 50 × 4.6 mm, stored in 20% (vol/vol) methanol (Phenomenex, cat no. 00B-4441-E0)
- Thin-layer chromatography (TLC) silica gel aluminum sheet, 20 × 20 cm (Millipore, F_{254}, cat. no. M1055540001)
- AKTA purifier 10 FPLC system, equipped with pump P-900, monitor pH/C-900, Box-900, injection valve INV-907, PV-908, mixer M-925, fraction collector Frac-950 and Unicorn 5.11 workstation (Glocal Medical Instrumentation, cat. no. 28-4062-64)
- MonoQ 4.6/100 PE anion-exchange column (GE Healthcare Life Sciences, cat. no. 17-5179-01)
- Superdex 200 Increase 10/300 GL size-exclusion chromatography column (GE Healthcare Life Sciences, cat. no. 28990944)
- AFM system (Bruker, Multimode 8 with Nanoscope V controller)
- MALDI-TOF system (Bruker, Autoflex)
- ScanAsyst-Fluid+ AFM probes, k ~0.7 N/m, tip radius <10 nm (Bruker)
- Software for designing DNA nanostructures (Tiamat, http://yanlab.asu.edu/Resources.html; cadnano, http://cadnano.org/; and Nanorex, http://nanorex-inc.software.informer.com/)
- Origin (v9.1) software (http://www.OriginLab.com)
- Transmission electron microscope (JEOL, model no. JEM-1400)
- Copper mesh grids (Electron Microscopy Sciences, cat. no. FCF400-CU-50)
- Parafilm M (Electron Microscopy Sciences, cat. no. 70990)
- Fine pointed tweezers (Dumont, cat. no. 72870-DZ)
- VIAFLO II Electronic Pipettes (Integra, single-channel, part no. 4011; 12-channel, part no. 4622)
- Quartz microscope slides, 1 inch × 3 inch × 1 mm (G. Finkenbeiner)
- Diamond-coated 'stick' drills, 1.0 mm (Shor International, cat. no. DIB-551.00)
- Coverslip, 4 × 30 mm (VWR, cat. no. 48404-466)
- Double-sided adhesive tape, 1/2 inch (3M Scotch, cat. no. 3136)
- Tubing (Cole-Parmer, cat. no. EW-06419-01)
- Costar Spin X filter, 0.22-μm cellulose acetate membrane (Corning, cat. no. CLS8160-96EA)
- Acrodisc syringe filter, 0.22-μm (Pall Life Sciences, cat. no. PN4602)
- 15-ml Conical centrifuge tubes (Thermo Fisher Scientific, cat. no. 339650)
- RA Lamb Glass Coplin Jar (Thermo Fisher Scientific, cat. no. 12608596)
- Emitech K100× machine (Quorum Technologies)

REAGENT SETUP

▲ **CRITICAL** All buffer solutions should be prepared in deionized water (18.2 MΩ · cm at 25 °C) and stored at 4 °C in the dark, unless stated otherwise. Buffer solutions should generally be replaced with fresh ones within one or two months.

50× TAE stock solution 50× TAE stock solution is 2 M Trizma base, 1 M acetic acid and 0.1 M EDTA. Combine 242.2 g of Trizma base, 57.1 ml of acetic acid and 37.2 g of EDTA.Na_2·12H_2O. Next, add dI H_2O to a total volume of 1,000 ml.

10× TAE–Mg^{2+} stock solution 10× TAE–Mg^{2+} stock solution is 0.4 M Trizma and 125 mM Mg^{2+}. Add 200 ml of 50× TAE stock to 26.8 g of $(CH_3COO)_2Mg{\cdot}4H_2O$. Add dI H_2O to a total volume of 1,000 ml. Adjusted the pH to 8 using NaOH or acetic acid. The solution should be filtered with a 0.22-μm filter.

1× TAE–Mg^{2+} buffer solution Dilute 10× TAE–Mg^{2+} stock solution to a final concentration of 0.04 M Trizma and 12.5 mM Mg^{2+}.

10× TBE stock solution 10 × TBE stock solution is 0.89 M Trizma, 0.89 M boric acid and 20 mM EDTA. Combine 108 g of Trizma base, 55 g of boric acid and 40 ml of 0.5 M EDTA, and then add dI H_2O to a total volume of 1,000 ml.

20% (vol/vol) denaturing PAGE gel mix A 20% Denaturing PAGE gel mix A is 20% acrylamide, 8.3 M urea and 1× TBE. Combine 500 ml of 40% acrylamide solution, 100 ml of 10× TBE and 500 g of urea. Next, add dI H_2O to a total volume of 1,000 ml. Store the solution at room temperature and protect it from light.

0% (vol/vol) Denaturing PAGE gel mix B Combine 500 g of urea and 100 ml of 10× TBE, and then add dI H_2O to a total volume of 1,000 ml. The final concentration of urea should be 8.3 M and the final concentration of TBE should be 1×. Store the solution at room temperature for up to 1 year and protect it from light.

0–20% (vol/vol) Denaturing PAGE gel 0–20% Denaturing PAGE gel is prepared by mixing 20% PAGE solution with 0% PAGE solution, as described in **Table 6**.

TABLE 7 | NaCl gradient for anionic-exchange FPLC of DNA-conjugated enzymes.

Step	Volume (ml)	% B (1 M NaCl)	Flow rate (ml/min)	Max. press. (MPa)
1	0	0	1	3
2	5	0	1	3
3	20	20	1	3
4	70	55	1	3
5	75	100	1	3
6	85	100	1	3
7	90	0	1	3
8	100	0	1	3

B, solvent B; Max. press., maximum pressure.

2× Denaturing tracking dye buffer, xylene cyanol FF (heavy one), 100 ml Prepare by mixing 90 ml of formamide, 37 mg of EDTA·Na_2·$2H_2O$, 40 mg of NaOH and 0.1 g of xylene cyanole FF dye to obtain a 0.10% dye concentration. Store the solution at room temperature for up to 1 year and protect it from light.

2× Denaturing tracking dye buffer, bromophenol blue (light one), 100 ml Prepare by mixing 90 ml of formamide, 37 mg of EDTA·Na_2·$2H_2O$, 40 mg of NaOH and 0.1 g of bromophenol blue dye to obtain a 0.10% dye concentration. Store it at room temperature for up to 1 year and protect it from light.

Denaturing PAGE elution buffer Denaturing PAGE elution buffer is 500 mM CH_3COONH_4, 10 mM $(CH_3COO)_2Mg$ and 2 mM EDTA. Prepare by combining 19.3 g of ammonium acetate, 1.07 g of magnesium acetate and 2 ml of 0.5 M EDTA. Add dI H_2O to a total volume of 500 ml with a pH of ~8.

10× Native tracking dye, 100 ml Prepare 10× native tracking dye by adding 0.20 g of bromophenol blue dye, 0.20 g of xylene cyanol FF dye, 50 ml of glycerol, 10 ml of 10× TAE–Mg^{2+} stock solution and 40 ml of dI H_20. Store the solution at room temperature for up to 1 year and protect it from light.

50 mM 6AE-NAD$^+$ solution Prepare 5 µmole (~3.5 mg) of 6AE-NAD$^+$ in dI H_2O to a total volume of 100 µl. Freeze the solution at –20 ° C for storage.

DNA-loading buffer for NAD$^+$–DNA conjugation DNA-loading buffer contains 10 mM acetic acid and 0.005% (vol/vol) Triton X-100. Combine 286.5 µl of acetic acid and 25 µl of Triton X-100, and then add dI H_2O to a total volume of 500 ml.

DNA elution buffer for NAD$^+$–DNA conjugation This buffer contains 50 mM HEPES (pH 7.5) and 1.5 M NaCl. Combine 1.3 g of HEPES sodium salt and 8.766 g of NaCl, and then add dI H_2O to a total volume of 100 ml. Adjust the pH to ~7.5 using 1 M HCl.

20 mM SPDP solution Prepare 1 mg of SPDP in DMSO to a total volume of 160 µl, and store the solution on ice. Prepare fresh SPDP before use.

20 mM T-CEP solution Prepare 1 mg of T-CEP in 10× TBS buffer to a total volume of 174.4 µl. Adjust the pH to ~7.5 using NaOH.

100 mM TEAA solution Prepare 100 ml of 1M TEAA stock in dI H_2O to a total volume of 1 L. The solution should be filtered with a 0.22-µm filter before use.

1 M NaH_2PO_4 stock Prepare 137.99 g of sodium phosphate monobasic monohydrate in dI H_2O to a total volume of 1 L.

1 M Na_2HPO_4 buffer stock Prepare 268.07 g of sodium phosphate dibasic heptahydrate in dI H_2O to a total volume of 1 L.

1 M sodium phosphate stock (pH 7.4) Combine 22.6 ml of 1 M NaH_2PO_4 and 77.4 ml of 1 M Na_2HPO_4 buffer stock. Add dI H_2O to a total volume of 1 L.

FPLC buffers Solvent A is 50 mM sodium phosphate (pH ~7.4). Add 50 ml of 1 M sodium phosphate (pH 7.4) to dI H_2O to a total volume of 1 liter. Solvent B is 50 mM sodium phosphate (pH ~7.4) + 1 M NaCl. Combine 50 ml of 1 M pH 7.4 sodium phosphate and 58.44 g of NaCl. Add dI H_2O to a total volume of 1 liter. Adjust the pH to 7.4 using NaOH or HCl. FPLC buffer solutions need to be filtered with a 0.22-µm filter before use to remove precipitates. It is strongly suggested to prepare FPLC buffer solutions freshly before use.

1 M Tris stock (pH 8.0) Dissolve 121.14 g of Tris base in 500 ml of dI H_2O. Adjust the pH to 8.0 using HCl. Add dI H_2O to a total volume of 1 liter.

1 M NaCl stock Dissolve 58.44 g of NaCl in 500 ml of dI H_2O. Add dI H_2O to a total volume of 1 liter.

10× T50 buffer (pH 8) 10× T50 buffer contains 0.1 M Tris, 0.5 M NaCl and 10 mM EDTA. Combine 0.5 ml of 1 M Tris-HCl (pH 8.0) and 2.5 ml of 1 M NaCl. Add dI H_2O to a total volume of 5 ml and filter the solution with a 0.22-µm syringe filter.

60% (wt/vol) PEG8000 stock First dissolve 3.0 g of PEG8000 in 3 ml of dI H_2O, and then adjust the final volume to 5 ml by adding dI H_2O.

1 M $MgCl_2$ stock First dissolve 2.033 g of $MgCl_2$·$6H_2O$ in 8 ml of dI H_2O, and then add dI H_2O to a total volume of 10 ml.

1× TBS–Mg^{2+}–PEG buffer (pH 7.5) 1× TBS–Mg^{2+}–PEG buffer contains 1× TBS, 1 mM Mg^{2+} and 10% (wt/vol) PEG8000. Combine 30 µl of 10× TBS (pH 7.5, purchased), 0.3 µl of 1 M $MgCl_2$ and 50 µl of 60% (wt/vol) PEG8000. Add dI H_2O to a total volume of 300 µl.

1× Oxygen-scavenging system 1× oxygen-scavenging system (OSS) is 50 nM PCD, 5 mM protocatechuic acid (PCA) and 2 mM Trolox[28,33]. 4 µM PCD stock is prepared in 50% (vol/vol) glycerol. 100 mM PCA stock is prepared in dI H_2O by adjusting the pH to ~9 using NaOH. Trolox is prepared by first dissolving the solids in methanol, followed by the addition of dI H_2O and NaOH (~pH 9.5, final methanol concentration should be ~10% (vol/vol)). The stocked solution of PCD is stored at 80 °C, and PCA and Trolox are stored at 20 °C until needed (typically for up to 3 months).

PEG reaction solution PEG solution is prepared by mixing 0.0252 g of Biotin-PEG-SVA and 0.084 g of mPEG-succinimidyl valerate and dissolving the mixture in 384 µl of 0.1 M sodium bicarbonate. The solution should be vortexed and centrifuged at 9,400*g* for 1 min at room temperature. to remove bubbles. ▲ **CRITICAL** PEG-reagent bottles can be stored at –20 °C for up to 6 months. Let the reagent bottle first warm up to room temperature before opening it to prevent condensation.

DST solution DST solution is 0.01 g of DST in 350 µl of 1 M sodium bicarbonate. Prepare the solution immediately before use.

100 mM $NiCl_2$ solution Prepare 238 mg of nickel (II) chloride hexahydrate in dI H_2O to a total volume of 10 ml.

0.5 M EDTA Mix 186.1 g of EDTA solid and 24 g of NaOH in dI H_2O to a total volume of 1,000 ml with pH ~8.

50 mM Sodium HEPES buffer (pH 7.5) Mix 13 g of sodium HEPES in dI H_2O to a total volume of 1,000 ml. Adjust the pH to ~7.5 using HCl.

1 M Sodium bicarbonate Mix 0.84 g of sodium bicarbonate in dI H_2O to a total volume of 10 ml. Vortex the mixture to dissolve solid sodium bicarbonate at room temperature. Prepare 0.5-ml aliquots and store them at −20 °C.

EQUIPMENT SETUP

PAGE setup The vertical electrophoresis system is used for the denaturing PAGE purification of oligonucleotides and the native PAGE characterization of DNA nanostructures. The sandwiched gel is assembled with two glass plates, two spacers on the sides and one spacer mate sheet in the middle. The sandwich is then secured with clamps and set upright on the gel casting stand and is well-sealed. The spacer mate will be removed before gel casting. The gel casting assembly should be left on the bench at room temperature for ~30 min for the gel to polymerize. The upper buffer chamber is first attached to the gel assembly and filled with running buffer. Buffer in the lower chamber should be the same as used in the upper chamber and can be stirred. The lower buffer chamber will be maintained at a certain temperature by water tubes that connect to a water-circulating bath. Power supply leads should be connected to a safety lid that covers the gel box. A constant current of 40 mA per gel and a 40 °C water bath for denaturing the PAGE gel are generally used. For native PAGE gel, a constant voltage of 200 V and 15 °C water bath are recommended.

FPLC setup Methods can be created, viewed and edited in the Unicorn workstation software. A flow rate of 1.0 ml/min should be used for anion-exchange chromatography, and a rate of 0.5 ml/min should be used for SE chromatography. The pump pressure limit should be set at 3–4 MPa for the protection of separation columns. An appropriate elution gradient should be

TABLE 8 | HPLC gradient for separating NAD+-modified DNA molecules.

Step	Time (min)	% B (methanol)	Flow rate (ml/min)	Max. press. (bar)
1	0	10	1	400
2	10	25	1	400
3	50	35	1	400
4	53	100	1	400
5	58	100	1	400
6	60	10	1	400

B, solvent B; Max. press., maximum pressure.

loaded into the system, as shown in **Table 7**. The flow system and the separation columns should be stored in 20% (vol/vol) ethanol if they are not used.

HPLC setup HPLC (Agilent 1200 series) should be used for purifying NAD+-modified oligonucleotides with an elution gradient from 25% (vol/vol) methanol/100 mM TEAA to 35% (vol/vol) methanol/100 mM TEAA, as shown in **Figure 4b**. The flow rate should be set at 1.0 ml/min. The pressure limit should be set at 400 bars. The HPLC flow system and the separation column should be stored in 10% (vol/vol) methanol while not in use. An appropriate elution gradient should be loaded into the system, as shown in **Table 8**.

AFM setup Assembled DNA origami samples with enzymes can be visualized and characterized using a Bruker Multimode 8 system with a Nanoscope V controller in a ScanAsyst in Fluid mode, which can be selected in the Nanoscope 8 software. The sample is imaged with the ScanAsyst-Fluid function mode, and the ScanAsyst-Fluid + AFM probes are equipped. Samples should be deposited on a freshly peeled mica surface and incubated with 1× TAE Mg^{2+} buffer (pH 8.0). The addition of 100 mM $NiCl_2$ can be used to enhance the adsorption of DNA nanostructures on the mica surface. To manually adjust the peakforce set-point, the autoscanning mode must first be switched off. Images can be viewed and processed off-line in the Nanoscope Analysis software.

smTIRF microscope setup As shown in **Figure 10**, the 532-nm He–Ne laser beam (532 nm, CrystaLaser CL532-050-L, 50 mW) first passes through a half-wave plate (multiorder plate to control the intensity of the beam), a polarizing beam splitter (PBS) and a dichroic mirror (610-nm cutoff)[28,33,49,84]. Another linearly polarized beam from a red diode laser (638 nm, Coherent CUBE 635-25C, 25 mW) passes through a clean-up filter (638 ± 10 nm) and is then reflected by a 610-nm cutoff dichroic (Chroma). The green and red laser beams are combined by the dichroic mirror, followed by passing through an iris diaphragm, a shutter, another iris diaphragm, a series of mirrors, a focusing lens and the prism for illuminating a microfluidic sample slide. The fluorescence from the sample is collected by a 1.2 numerical aperture 60× water-immersion objective (IX71, Olympus) in a darkened room at an environmentally controlled temperature of 20 ± 3 °C. The Cy3 and Cy5 fluorescence emission signals are separated by a dichroic mirror with a cutoff wavelength of 610 nm (Chroma) and projected side-by-side onto an intensified CCD (ICCD) camera chip (512 × 512 pixel, iPentamax HQ Gen III, Roper Scientific) with a full-frame acquisition rate of 10–100 Hz. The Cy3 channel image is passed through a band-pass filter (HQ580/60 nm, Chroma), and the Cy5 channel is passed through a long-pass filter (HQ655LP, Chroma). A Newport ST-UT2 vibration isolation table was used in all experiments to reduce instrument interference.

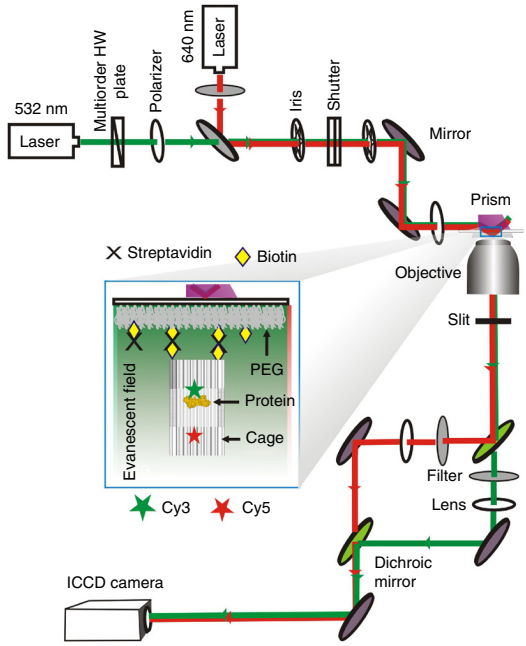

Figure 10 | Schematic of prism-based TIRF setup. 532-nm light from a HeNe laser passes through a l/2-wave plate (multiorder plate to control the intensity of light), polarizing beam splitter and a dichroic mirror. Linearly polarized light from the red diode laser (638-nm) passes through a cleanup filter (638 ± 10 nm) and is reflected by a 610-nm cutoff dichroic mirror. Beams from both green and red lasers are combined and then passed through an iris diaphragm, a shutter, another iris diaphragm, a series of mirrors, a focusing lens, the prism, the microscope slide containing the sample, the 60× objective, a 1.6× magnifier (along with additional filters, mirrors and image-transferring lenses contained within the microscope), a slit and a 610-nm-cutoff dichroic mirror. In our setup, the donor emission passes through a band-pass filter (HQ580/60 nm), and the acceptor emission passes through a long-pass filter (655 nm). These separated images are projected side-by-side onto an intensified CCD (ICCD) 512 × 512 pixel camera.

PROCEDURE

DNA–enzyme conjugation and purification ● TIMING 6–8 h

1| Prewash enzymes three times with 50 mM sodium HEPES buffer (pH 7.5) using an Amicon 30-kDa cutoff filter with the centrifuge at 3,234*g* for 15 min at 4 °C, which removes the small impurities and primary amine contaminates. Quantify the concentration of collected protein solution by UV-visible light absorbance using the NanoDrop. Prepare G6PDH, GOx, HRP, LDH and malate dehydrogenase (MDH) solutions as in our previous studies, in which ~500 µl of 5 mg/µl enzyme solution was added to a 4-ml Amicon 30-kDa filter tube, followed by the addition of 4 ml of 50 mM HEPES buffer (pH 7.5). Centrifuge the filter tube at 3,200*g* on a swing-bucket rotor for 15 min at 4 °C. Collect the remaining enzyme solution in the filter, and repeat the wash step twice. For a smaller quantity of enzyme solution, e.g., 100 µl, we use an Amicon 30-kDa cutoff filter (0.5 ml) and centrifuge the solution at 9,400*g* for 15 min at 4 °C.

! CAUTION You must wear gloves when handling biochemical reagents.

▲ CRITICAL STEP Before collecting the enzyme solution, use a micropipette to mix the solution a few times to resuspend the accumulated enzymes in the bottom of the filter.

2| Prepare 20 mM SPDP stock solution in DMSO, and store it on ice for <1 h (Reagent Setup). Add an appropriate amount of SPDP (2- to 20-fold excess, depending on enzymes) to the enzyme solution. For example, for a fivefold excess SPDP, 5 µl of SPDP stock solution should be added to 500 µl of 40 µM enzyme solution. Next, add 100 µl of 1 M $NaHCO_3$ to adjust the final pH value of the reaction solution to ~8.5. Place the reaction solution in a brown centrifuge tube or wrap the tube with aluminum foil, and incubate it on a rocker at room temperature for 1 h.
▲ **CRITICAL STEP** SPDP (NHS ester and pyridyldithiol) is not stable in water or DMSO. Prepare fresh SPDP before the conjugation reaction. The control of pH (8–8.5) is critical to the SPDP modification. We suggest using a pH paper (8–10) to test the pH value of the reaction mixture.

3| After the first step of the reaction, remove excess SPDP by centrifugal filtration with 50 mM HEPES (pH 7.5) buffer. To do this, use Amicon 30-kDa cutoff filters and centrifuge at 9,400*g* for 15 min at 4 °C, repeating three times. For a large quantity, use a swing-bucket rotor and centrifuge at 3,200*g* for 15 min at 4 °C. Collect the SPDP-modified enzymes and quantify the enzyme concentration with NanoDrop. Also record the absorbance at 343 nm.
▲ **CRITICAL STEP** SPDP-modified enzyme must be used for the DNA conjugation within a few hours. The modified SPDP group is not stable overnight.

4| Prepare a 20 mM T-CEP solution in 10× TBS buffer, and adjust the pH to ~7.5 (Reagent Setup). Add 1 µl of 20 mM T-CEP to 20 µl of SPDP-modified enzyme solution, and incubate the reaction solution in the dark at room temperature for half an hour. Quantify the labeled SPDP by reading the increased absorbance at 343 nm due to the release of pyridine-2-thione (extinction coefficient: 8,080 per M per cm).
▲ **CRITICAL STEP** The average number of labeled SPDP molecules per enzyme should be between 1 and 2 for maintaining the activity. If an enzyme is labeled with more than three SPDP molecules, the modified enzyme activity will be significantly damaged. Some enzymes may have a background absorbance at 343 nm (e.g., GOx or HRP). Thus, the increased absorbance can be estimated by $\Delta A_{343} = A_{343}$ (SPDP-enzyme + T-CEP) – A_{343} (SPDP-enzyme).
? TROUBLESHOOTING

5| The thiol-modified DNA purchased from IDT is protected by a disulfide bond. Before use for conjugation, add 20-fold excess T-CEP to the DNA solution and incubate the mixture for 1 h to cleave the disulfide bond. After the cleavage, remove the excess T-CEP by centrifugal filtration with water using an Amicon 3-kDa-cutoff filter (4 ml). Centrifuge at 3,200*g* on a swing-bucket rotor for 45 min at 4 °C, repeating three times. A successful cleavage can be characterized by MALDI-TOF. Collect the thiol-modified DNA and store it at –20 °C for up to ~1 month.
? TROUBLESHOOTING

6| Incubate the SPDP-modified enzyme solution with a tenfold excess thiol-modified DNA solution in 50 mM sodium HEPES (pH 7.5) for 1 h at room temperature in the dark. A successful conjugation can be evaluated by measuring the increased absorbance at 343 nm.

7| After the reaction, remove excess DNA molecules by centrifugal filtration using Amicon cutoff filters (e.g., 30 kDa). For 500 µl of DNA-conjugated enzyme solution, add 3,500 µl of 50 mM HEPES (pH 7.5) containing 1.5 M NaCl and wash twice, and then wash twice more with regular 50 mM HEPES (pH 7.5).

8| Quantify the concentration of the DNA-conjugated enzyme and the average DNA label ratio by UV-visible light absorbance of 260 nm and 280 nm, as discussed in the Experimental design.
▲ **CRITICAL STEP** If the measured number of labeled DNA molecules per enzyme is still higher than the average number of labeled SPDP molecules per enzyme, it indicates the presence of nonspecific and unconjugated DNA molecules in solution. An additional wash with 50 mM HEPES (pH 7.5) containing 0.05% (vol/vol) P20 is required to remove these nonspecifically bound DNA molecules.
■ **PAUSE POINT** The washed DNA-conjugated enzymes can be stored at 4 °C for up to 1–2 months.

AE-FPLC purification of DNA-conjugated enzymes ● TIMING 3–5 h
9| First, spin down the solution of DNA-conjugated enzyme at 9,400*g* for 5 min at 4 °C to remove the insoluble aggregates. Rinse the FPLC pumps, flow lines and separation column with dI H_2O at a flow rate of 1.0 ml/min for 10 min. Next, equilibrate the column with solvent A at a flow rate of 1.0 ml/min for 10 min. Load the supernatant enzyme solution into the FPLC system with an anion-exchange column, and then separate the sample by using an elution gradient from 20% (vol/vol) solvent B to 55% (vol/vol) solvent B, with a flow rate of 1.0 ml/min. The elution gradient is shown in

Table 7. After use, clean the FPLC system by flowing sequentially with 10 ml of solvent B and 30 ml of of dI H_2O. Then, store the column in a 20% (vol/vol) ethanol–water solution.
▲ **CRITICAL STEP** The sodium phosphate buffer must be prefiltered with a 0.22-μm filter in order to avoid any insoluble particles that may block the flow channel of the instrument.

10| Multiple peaks from the purification chromatogram should be collected and concentrated to an ~100-μl volume using an Amicon cutoff filter (e.g., 30 kDa). The DNA-conjugated enzyme should be characterized by UV-visible light absorbance for measuring the concentration and the labeled DNA ratio.
■ **PAUSE POINT** The purified DNA-conjugated enzyme solution can be stored at 4 °C for 4–8 weeks. To further increase its stability, add 40% (vol/vol) glycerol to the enzyme solution, and keep the frozen solution at –20 °C for up to 6 months.

DNA–NAD⁺ conjugation ● TIMING ~4 h
11| Prepare 10 mM amine-modified DNA and 50 mM AE-NAD⁺ in dI H_2O. Prepare the DNA-loading buffer and elution buffer (Reagent Setup) for conjugation (**Fig. 4a**).

12| Shake the bottle of DEAE resin vigorously to emulsify the resin solution. Add 200 μl of resin to a Sigma Prep column and spin it down at 376*g* for 30 s at room temperature to remove the solvent. Then, wash the resin three times with one column-bed volume (500 μl) of DNA-loading buffer.

13| Prepare 200 μl of 20 nmole DNA in DNA-loading buffer (10 mM acetate acid and 0.005% (vol/vol) Triton X-100) by mixing 2 μl of 10 mM amine-modified DNA stock with 198 μl of loading buffer. Add DNA to the resin and incubate the DNA–resin mixture on a vortex shaker for 10 min at room temperature, allowing the DNA to bind to the resin. The suggested ratio of DNA to resin is ~1 nmole DNA:10 μl resin. After the incubation, remove the excess solution by spinning down at 376*g* for 30 s at room temperature. DNA-binding efficiency can be calculated by measuring the 260-nm absorbance of the DNA solution before and after incubation with the resin: (A_{260} (before) – A_{260} (after))/A_{260} (before). The DNA-binding efficiency should be >90% for linear oligonucleotides.
▲ **CRITICAL STEP** The bottom of the spin column must be capped with red rubber during the incubation. To reduce DNA dimerization, a lower DNA-to-resin ratio of 1 nmole DNA per 20 μl resin may be used.
? TROUBLESHOOTING

14| Wash the resin with 500 μl of anhydrous DMF twice to remove extra water.

15| Prepare the DSS linker in DMF with 2% (vol/vol) DIPEA. For 20 nmole DNA, 200 μl of 150 mM DSS is needed. Add the DSS linker to the resin-bound DNA and incubate for 1 h on a vortex shaker at room temperature.
! CAUTION DMF is flammable, toxic on contact with skin and causes eye irritation. It is also known as a chemical that causes respiratory sensitization. DIPEA is flammable, corrosive and toxic.
▲ **CRITICAL STEP** The DSS linker is moisture-sensitive. Store the DSS chemicals in a desiccator at –20 °C. The DSS solution should be freshly prepared just before the reaction. The bottom of the spin column must be capped with red rubber during incubation.

16| Wash the reactant three times with 500 μl of dry DMF to remove excess DSS linker. Then wash it once with 500 μl of DNA-loading buffer to restabilize surface-bound DNA.
▲ **CRITICAL STEP** The resin-bound DNA must be restabilized by DNA-loading buffer before the addition of AE-NAD⁺.

17| Add tenfold excess of AE-NAD⁺ to the DSS-modified DNA. For 20 nmole DNA, 200 nmole AE-NAD⁺ is prepared by adding 4 μl of 50 mM NAD stock to 10 μl of 1 M HEPES (pH 8.0) and 186 μl of water (final reaction buffer contains 50 mM HEPES). Incubate the mixture on a vortex shaker at room temperature for 1 h.
▲ **CRITICAL STEP** The pH of the solution must be controlled (kept between 8 and 8.5) to improve the amine-NHS reaction efficiency. Avoid adding a high concentration of NaCl (<100 mM) to the reaction buffer to prevent any ionic elution of resin-bound DNA molecules. The bottom of the spin column must be capped with red rubber during incubation.
■ **PAUSE POINT** The mixture can be stored at 4 °C overnight.

18| Add 300 μl of DNA-loading buffer to the reaction mixture and incubate for 10 min to restabilize the DNA-bound resin.
▲ **CRITICAL STEP** The bottom of the spin column must be capped with red rubber during incubation.

19| Remove the red rubber cap and spin down the solution at 376*g* for 30 s at room temperature to remove excess AE-NAD^+. Wash the resin with 200 µl of elution buffer (50 mM HEPES (pH 7.5) and 1.5 M NaCl) three times and collect the filtrate.
■ **PAUSE POINT** The filtrate can be stored at 4 °C overnight.

20| Wash and concentrate the eluted solution three times with dI H_2O using an Amicon 3kDa filter. The washed solution should be ready for HPLC purification.
■ **PAUSE POINT** The washed solution can be stored at 4 °C for several days.

HPLC purification of NAD^+-conjugated DNA ● TIMING 1–2 d

21| Set up the HPLC system and running protocol as described in the Equipment Setup. Rinse the flow system with dI H_2O to remove the stored solvent.

22| Attach a MonoQ 4.6/100 PE anion-exchange column to the HPLC system. Rinse the column with 10 column volumes of water at 1 ml/min to remove the stored solvent (e.g., 10% (vol/vol) methanol). Then, switch the solvents to purge pump A and pump B with running buffers of TEAA and methanol, respectively, at a flow rate of 2 ml/min for 5 min. Switch the line to connect with the column and equilibrate it with flowing 90% (vol/vol) TEAA and 10% (vol/vol) methanol for at least ten column volumes at 1 ml/min.
! CAUTION TEAA may cause eye and skin irritation. Methanol is flammable and toxic, and can cause respiratory sensitization.
▲ **CRITICAL STEP** During the pump purge, make sure that the column is disconnected from the flow lines. Before loading the sample, the flow pressure must be stabilized during the column equilibration.

23| Inject 5 µl of DNA–NAD^+ conjugation mix from Step 19 for an analytical run. It should be used to optimize the solvent gradient and sample collection in **Table 8**.

24| Inject no >100 µl of sample, and run the HPLC system for separation and collection.
▲ **CRITICAL STEP** For sample collection, it should be realized that there is a delay time between the detector and the collector. Set up the appropriate delay for accurately collecting separated samples. The delay time can be estimated by injecting a dye solution into the HPLC system without the column and observing the elution of the sample.

25| Transfer the collected samples to 15-ml centrifuge tubes with a cap and freeze them at –80 °C for 1 h. Drill a few tiny holes into the cap and dry the samples overnight using the lyophilizer. Re-dissolve the lyophilized samples in 50–100 µl of dI H_2O and quantify the concentration with 260-nm absorbance.
■ **PAUSE POINT** The purified DNA-NAD^+ solution can be stored at –20 °C for up to several months.

26| Use MALDI-TOF to characterize the purity and molecular weight of a DNA-NAD^+ sample (**Fig. 4b**; **Box 1**).
? TROUBLESHOOTING

Denaturing PAGE purification of the oligonucleotides ● TIMING 2 d

▲ **CRITICAL** Oligos used to create DNA nanostructures should be designed using open-access software (Tiamat[19] or cadnano[17]).
27| Prepare a gel mixture as suggested by **Table 6**, adding together denaturing PAGE gel mixes A and B to a desired concentration.
! CAUTION Acrylamide:bis-acrylamide solution is highly toxic and is a potential human carcinogen and teratogen. It is also known as a potent neurotoxin. Wear protective gloves and clothes. All the waste solution and pipettes must be collected in special containers. It is recommended to assign a special lab space for operating PAGE gels; this should be labeled 'PAGE contamination area'.

28| Add 263 µl of APS and 14.7 µl of TEMED to imitate the polymerization of the gel. Carefully load 35 ml of the mixture solution into the gel cassette using a 50-ml transfer pipette.
▲ **CRITICAL STEP** To ensure a uniform electrical conductance, make sure that there are no air bubbles trapped in the gel during pouring.

29| Insert a comb with 15 wells. The gel casting assembly should be left on the bench at room temperature for ~60 min for the gel to be polymerized. Then, remove the comb carefully and rinse the wells with dI H_2O.
? TROUBLESHOOTING
■ **PAUSE POINT** The gel can be stored at 4 °C for up to 1 month. To avoid drying, it is recommended to wrap gels with plastic film.

30| Clamp the gel to the electrophoresis tank and carefully rinse the bottom of the wells with 1× TBE buffer using a syringe.
▲ **CRITICAL STEP** The bottoms of the gel wells must be cleaned with buffer to remove any precipitates such as urea crystals or gel aggregates; otherwise, the DNA sample may not completely diffuse into the gels.

31| Prepare the oligonucleotides in dI H_2O with a concentration of ~0.5 optical density units (OD)/μl. Add an equal volume of 2× denaturing tracking dyes (Reagent Setup; bromphenol blue or xylene cynole FF, or both).
▲ **CRITICAL STEP** To improve the efficiency of PAGE purification, 5 OD of oligonucleotides should be loaded into each well of the gel. For tracking dyes, avoid using a dye with mobility close to that of the oligonucleotides; otherwise, it may contaminate the DNA sample. The mobility of tracking dyes is illustrated in **Table 6**.

32| Vortex the mixture for 10 s, and spin it down at 376*g* for 30 s at room temperature. Next, heat the mixture to 90 °C for 5 min.
▲ **CRITICAL STEP** Do not heat the sample to a higher temperature or for a longer time.

33| Load ~20 μl of the sample mixture into each well, with ~5 OD per lane.

34| Turn on the water-circulating bath and set the temperature to ~35 °C. Run the gel at a constant current of ~45 mA for ~1 h. Use the tracking dyes to estimate the migration distance of the sample.
! **CAUTION** High voltage.

35| After the electrophoresis, place the gel on a UV transilluminator in a dark room. With 254-nm UV light, the DNA band can be directly visualized without the addition of staining dyes (e.g., ethidium bromide). Cut the DNA band from the gel using a razor blade and chop it into small pieces.
! **CAUTION** UV radiation is hazardous and ethidium bromide is toxic. Wear a UV-protection shield, gloves and lab coat during the operation under UV. Avoid wearing any shiny objects such as rings to prevent any unexpected reflection of the UV beam.
▲ **CRITICAL STEP** Prolonged UV illumination may damage the DNA, for instance, causing thymine dimerization.
? **TROUBLESHOOTING**

36| Collect the small pieces of DNA gel using a centrifugal filter tube (Costar Spin X filter, 0.22 μm). Add 500 μl of denaturing PAGE elution buffer, and incubate the mixture on a shaker overnight.
■ **PAUSE POINT** The elution process can be prolonged for 3 d (e.g., over the weekend).

37| Centrifuge the filter tube at 9,400*g* for 5 min at room temperature; discard the gel blocks on the top of the filter. Add 1,000 μl of ethanol to the filtrate solution and vortex to mix it. Incubate the mixture in a –80 °C freezer for 1 h.
▲ **CRITICAL STEP** Shorter strands are harder to precipitate and need a longer time for incubation.

38| Centrifuge the mixture at 9,400*g* for 30 min at 4 °C. This step is to precipitate the DNA. Check whether there are white precipitates on the bottom of the tube. Carefully pour out the liquid. Then add 1,000 μl of ice-cold 70% (vol/vol) ethanol to the precipitates, mix well and centrifuge again at 9,400*g* for 10 min at 4 °C. Pour out the solution.

39| Use a Vacufuge to dry the DNA sample at 30 °C for 3–6 h. Then add in 50 μl of dI H_2O to dissolve the DNA solids, and vortex the mixture for 1 min. The concentration of DNA should be measured by absorbance at 260 nm using a NanoDrop.
■ **PAUSE POINT** The DNA solutions (both purified and crude) can be stored at 4°C for a few days. It is best to store at –20 °C for long-term storage (several months).
? **TROUBLESHOOTING**

Assembly of DNA nanostructures ● **TIMING 2–24 h**
40| The assembly and purification of DNA origami nanostructures should be performed using the following procedures. For assembly of DX-DNA tiles, follow option A; for assembly of DNA origami structures, follow option B.
(A) Assembly of DX-DNA tiles
(i) Purify all oligonucleotides using 8% denaturing PAGE, as described above in Steps 27–39. Prepare a 100-μl sample solution by mixing all oligonucleotides together with 1 μM of each strand in 1× TAE–Mg^{2+} buffer (pH 8.0).
(ii) Anneal the DNA mixture using a PCR thermocycler (Eppendorf) from 90 °C to 4 °C with the temperature gradient as shown in **Table 3**.
▲ **CRITICAL STEP** The volume of DNA solution cannot exceed 100 μl per PCR tube; otherwise, the temperature will not be precisely controlled during the thermal annealing process. Use ice to cool the cap of the PCR tube before taking the sample tube out of the PCR plate.
? **TROUBLESHOOTING**
■ **PAUSE POINT** The assembled DNA structures can be stored at 4 °C for a few weeks.

(B) Assembly of DNA origami structures

(i) Single-stranded staples should be ordered from IDT without further purification. The capture strands should be purified using an 8% denaturing PAGE, as described above.
▲ **CRITICAL STEP** IDT provides staple strands (~100–200 stands) in 96-well plates with 100 μM in water. Prepare 1-μM core solutions by mixing together all the staple strands in the same 96-well plate, and label as core 1, core 2 and core 3. The capture strands are excluded from the core solutions.

(ii) Mix 100 μl of 20 nM single-stranded M13mp18 DNA with a fivefold molar excess of staple stands and a tenfold molar excess of capture strands in 1× TAE–Mg^{2+} buffer (pH 8.0)[25].

(iii) Anneal the DNA mixture using a PCR thermocycler from 90 °C to 4 °C with the temperature gradient as shown in **Table 3**.
▲ **CRITICAL STEP** The assembly of 3D DNA origami structures requires a longer and slower annealing program as compared with the assembly of 2D DNA origami structures.
■ **PAUSE POINT** The assembled DNA origami structures can be stored at 4 °C for a few weeks. Do not remove the excess staple and capture strands, as they enhance the stability of the assembled structures.

(iv) Remove excess staple and capture strands by the centrifugal filtration of the origami solution with an Amicon 100-kDa filter using 500 μl of 1× TAE–Mg^{2+} buffer (pH 7.5). Repeat this step three times. Quantify the concentration of the DNA origami solution by 260-nm absorbance using an estimated extinction coefficient of ε_{260} ~110,000,000 per M per cm.

(v) Analyze the purity of the origami tiles by AGE (2%), as shown in **Box 3**.

Assembly of enzymes onto DNA nanostructures ● TIMING 2 h

41| Incubate 100 μl of the DNA nanostructure solution with a threefold excess DNA-conjugated enzyme solution in 1× TAE–Mg^{2+} buffer (pH 7.5). The mixture should be thermally incubated within a PCR thermocycler with a temperature gradient from 37 to 10 °C (refs. 25,28,29,33). The detailed temperature gradient is 37 °C for 5 min; 36–10 °C, 2 min per degree decrease. The solution is then kept at 4 °C for storage. After the assembly, the enzyme-assembled DNA nanostructures should be visualized and characterized using gel electrophoresis, AFM or TEM.
■ **PAUSE POINT** The assembled enzyme–DNA nanostructures can be stored at 4 °C for up to 2–3 weeks. However, it is strongly recommended to use the sample within a week.

Characterization of enzyme-assembled DNA nanostructures ● TIMING 2–24 h

42| Multiple tools can be used to characterize the enzyme-assembled DNA nanostructures and to estimate the yield of assemblies, including gel electrophoresis (option A), AFM/TEM (option B) and smTIRF (option C). For enzymes on smaller DNA tiles, follow option A; for enzymes on DNA origami structures, follow option B. Option C (smTIRF) can be used to further accurately quantify the yield of enzyme-assembled DNA nanostructures, especially for DNA nanocages, for which encapsulated enzymes cannot be clearly visualized by AFM/TEM.

(A) Gel electrophoresis characterization of enzyme-assembled DNA nanostructures ● TIMING 6–8 h

(i) For small DNA DX tiles, use 3–5% native PAGE to characterize the enzyme assemblies, as described in **Box 2**. For DNA origami structures, 2% AGE is used to characterize the enzyme-assembled structures, as shown in **Box 3**.
▲ **CRITICAL STEP** For large DNA origami nanostructures, it is almost impossible to differentiate the band shift between the DNA nanostructures and the enzyme-assembled DNA nanostructures. Thus, fluorescently labeled enzymes and DNA nanostructures are used for dual-color gel imaging to confirm the assembly of enzymes onto the DNA structures, as shown in **Figure 5a**,**b**. The fluorescently (e.g., Cy3 or Cy5) labeled oligonucleotides can be purchased from IDT.
? TROUBLESHOOTING

(B) AFM/TEM characterization of enzyme-assembled DNA nanostructures ● TIMING 1–2 h

(i) For the direct observation of enzyme assembly on DNA nanostructures, use AFM to visualize small DNA DX tiles and 2D DNA origami structures, and use TEM to image 3D DNA nanostructures. Protocols to do this are described in **Box 4**. The assembly yield should be estimated by counting the enzyme-assembled DNA nanostructures and the DNA nanostructures containing no enzymes.
? TROUBLESHOOTING

(C) smTIRF characterization of DNA nanocaged enzymes ● TIMING 17–21 h

(i) *Preparation of PEGylated slides for smTIRF (7–8 h)*. Drill a new quartz microscope slide with two 1-mm holes at the two ends of a diagonal line; these can be covered by a coverslip. Next, rinse the slide thoroughly with water and acetone, and dry the slide using pure nitrogen gas[84,85].

(ii) The aminosilyation of the surface should be performed by adding 70 ml of aminosilation mixture to a Coplin jar containing five quartz slides. Prepare the aminosilation mixture by adding together 2 ml of APTES and 98 ml of acetone. The Coplin jar should first be incubated at room temperature for 10 min, followed by sonication for 1 min; then incubate it for another 10 min.
▲ **CRITICAL STEP** APTES should be stored at 4°C for up to 1 month.

(iii) After the incubation, dispose of the APTES solvent, rinse the slides thoroughly with dI H_2O, and dry with nitrogen gas.
(iv) Perform PEGylation of the slide surface by incubating a slide with 70 µl of PEG reaction solution in 0.1 M sodium bicarbonate.
▲ **CRITICAL STEP** Prepare this solution immediately before adding it to the slides.
(v) Carefully place a coverslip onto the top of the solution on the slide. Incubate the slide at room temperature for 2–4 h.
▲ **CRITICAL STEP** Avoid trapping air bubbles between the coverslip and the microscope slide.
(vi) Rinse the slide again with water and dry the slide completely with nitrogen gas.
(vii) To cap the unreacted amine groups on the aminosilyated slide surface, add 70 µl of DST solution onto a PEGylated slide, and cover it with a coverslip. Incubate the DST solution with the slide at room temperature for 30 min.
▲ **CRITICAL STEP** Prepare DST solution immediately before use.
(viii) Rinse the slide thoroughly with dI H_2O, and dry it completely with nitrogen gas.
(ix) To assemble the slide for imaging, a flow channel connecting the two drilled holes should be created by using two pieces of double-sided tape to seal the coverslip to the PEGylated slide, leaving an ~2- to 3-mm channel in the middle[84]. Then, seal the edges of the slides with epoxy glue. These PEGylated slides may be stored within an evacuated desiccator (filled with nitrogen) in the dark at 4 °C for up to 2 weeks.
(x) *smTIRF characterization of DNA nanocaged enzymes (10–13 h)*. Estimate the encapsulation yield by the fluorescence colocalization of the Cy3-labeled enzyme and the Cy5-labeled DNA cage, as described in **Box 5**.
? TROUBLESHOOTING
(xi) Process the data for single-molecule fluorescence colocalization using the custom-written scripts in IDL and MATLAB. The detailed procedures for image processing and analysis are described in the **Supplementary Methods**.
(xii) Process hidden Markov model (HMM) fitting of single-channel fluorescence traces for counting the stepwise photobleaching events. The detailed procedures for image processing and analysis are described in the **Supplementary Methods**.

Purification of enzyme-assembled DNA nanostructures ● TIMING 4–6 h

43| Two methods can be used to purify enzyme-assembled DNA nanostructures, including SE chromatography (option A) and AGE (option B). For enzymes assembled on small DNA DX tiles, follow option A; for enzymes assembled on DNA origami structures, follow option B.

(A) SE purification of enzyme-assembled DNA nanostructures ● TIMING 4 h

(i) For a typical purification, load ~500 µl of 750 nM enzyme–DNA assemblies onto the FPLC system and elute with 100 mM HEPES (pH 8) at a flow rate of 0.5 ml/min. An example chromatogram for purification of enzyme-assembled DNA DX tiles is shown in **Figure 6a**.
▲ **CRITICAL STEP** The SE column of the Superdex 200 Increase 10/300 GL is a low-pressure column (maximum pressure ~4 MPa). Adjust the flow rate to lower the pressure of the system.
(ii) Estimate the concentration of the purified enzyme–DNA tiles at 260-nm absorbance using an extinction coefficient that is equal to the ε_{260} sum of the double-stranded DNA and the enzymes.

(B) AGE purification of enzyme-assembled DNA origami structures ● TIMING 4–6 h

(i) For enzyme-assembled DNA origami structures, remove any excess and free enzymes using AGE, as described in **Box 3**. The concentration of purified structures is estimated using the extinction coefficient of DNA origami with ε_{260} ~110,000,000 per M/per cm. The absorbance of individual enzymes is negligible as compared with the large extinction coefficient of DNA origami.

Evaluation of enzyme activity ● TIMING 2–13 h

44| Enzyme activities can be determined in bulk solution (option A) or measured by single-molecule fluorescence (option B). Users can choose option A, or option B or both to characterize the activity of enzyme-assembled DNA nanostructures.

(A) Enzyme activity assay in bulk solution ● TIMING 2 h

(i) Perform the enzyme assay using a 96-well microplate reader (e.g., Multimode from Molecular Devices or BioTek Cytation 3). Set the instrument ready for kinetic measurements of absorbance or fluorescence. For a typical assay, prepare all substrates in 1× TBS buffer (pH 7.5) with 1 mM $MgCl_2$. Add 50 µl of enzyme–DNA assemblies to each well first. Next, transfer the substrate to a 96-well plate with 50 µl of solution per well to initiate the reaction, and start the reading immediately.
▲ **CRITICAL STEP** It is strongly recommended to use electronic multiple-channel pipettes to increase the pipetting accuracy between multiple samples. Enzyme assays should be performed with at least three replicates.
(ii) Use the kinetical software carried by the instrument, or other data-analyzing software (e.g., GraphPad Prism or Origin) to fit the initial velocity of the enzyme-catalyzed reactions. Detailed kinetical analysis includes the measurement of Michaelis–Menten curves and standard curves of product molecules.

(B) Single-enzyme activity assay ● TIMING 10–13 h

(i) Immobilize the enzyme-encapsulated DNA nanocages on the PEGylated slides via biotin–streptavidin interaction and analyze using smTIRF microscopy, as described in **Box 5**.
▲ **CRITICAL STEP** 1× OSS solution should be added to the sample solution to prevent photobleaching from the laser illumination. For 300 µl of sample, add 7.5 µl of 4 µM PCD, 7.5 µl of 100 mM PCA and 3 µl of 100 mM Trolox to reach a final concentration of 1× OSS in the sample solution. Incubate the solution for 2–3 min for the OSS to decrease the oxygen level in the buffer. If photobleaching is still pronounced in 1× OSS, add 2× OSS to the sample and incubate for 2–3 min.

(ii) To initiate the enzyme reaction, inject 300 µl of substrate solution (1 mM G6P, 1 mM NAD^+, 50 nM resazurin and 12.5 µM PMS in 1× TBS–Mg^{2+}–PEG buffer (pH 7.5, 1 mM Mg^{2+} and 10% (wt/vol) PEG8000)) into the flow channel. After injecting the substrate solution, movies should be recorded for ~5 min (9,091 frames) at a 35-ms camera integration time.

(iii) Analyze recorded movies using custom-written scripts to examine fluorescent trajectories and spikes. For more detailed information on the scripts, please see the **Supplementary Methods**.

? TROUBLESHOOTING

For troubleshooting advice, please see **Table 9**.

TABLE 9 | Troubleshooting table.

Step	Problem	Possible reasons	Solutions
DNA–enzyme conjugation			
4	Enzymes are labeled with very few SPDP molecules	Buffer contains amine contaminants The pH of the buffer is neutral or acidic SPDP is hydrolyzed by moisture or is left too long on the bench Very few lysines are available on the enzyme surface	First wash enzyme solutions with nonamine buffers such as phosphates or HEPES Adjust the pH of the reaction solution to ~8–8.5 Prepare fresh SPDP in DMSO (anhydrous), and immediately add it to the enzyme solutions Using the Protein Data Bank, check the enzyme structures, and identify the surface lysines
5	Thiol-modified DNA reacts poorly with SPDP-modified enzymes	The disulfide-bond protection group is not fully cleaved T-CEP is not fully removed from the DNA solution SPDP-modified enzymes were stored for too long with a broken pyridyldithio group	Cleave the disulfide-bond protection group with T-CEP Completely remove T-CEP by centrifugal filtration with water or buffer three times Prepare fresh SPDP-modified enzyme, and use it immediately
NAD^+–DNA conjugation			
13	DNA-binding efficiency is low	There are folded secondary structures that prevent DNA from binding to the resin	Add 10% (vol/vol) DMSO to the DNA-loading buffer to open the folded structures
26, **Box 1**—step 9	The MALDI-TOF signal of DNA is very weak	Salt (e.g., Na^+) may be present in the sample solution 3HPA solution is not saturated DNA concentration is either too high or too low Laser intensity is not strong enough	Desalt the DNA solution Prepare saturated 3HPA by ultrasonic mixing Add a few more microliters of 3HPA to the 0.5 µl of DNA solution Prepare a solution containing from a few micromoles to 20 µM of DNA Increase laser intensity Increase pulsed extraction time Average more shots

(continued)

TABLE 9 | Troubleshooting table (continued).

Step	Problem	Possible reasons	Solutions
Denaturing PAGE			
29	Gel is not completely solidified after 60 min	APS loses activity during storage	Make sure that APS is kept at –20 °C, and prepare fresh APS solution
35	The sample band is very thick and wide (a round spot instead of a thin band)	Wells were overloaded with DNA samples	Collect the major band and repeat the gel purification with a smaller amount of DNA per lane
39	A_{260} value is low for recovered DNA sample	Ethanol precipitation was not successful because of the short freeze time (Step 37), or the DNA sample was left at room temperature for too long before pouring out the ethanol (Step 38)	Collect the ethanol waste and repeat from Step 37
Assembly of DNA nanostructures			
40A(ii)	Cofactor-modified DNA (e.g., NAD^+) loses activity after the thermal annealing process	Thermal damage and deactivation of cofactors	Decrease the maximal temperature of the thermal annealing program Limit the thermal incubation above 70 °C to <10 min
Native PAGE			
42A(i), **Box 2**—step 3	Gel is not completely solidified after 3 h	Low-percentage gel needs longer solidification time	Leave the gel overnight to solidify
42A(i), **Box 2**—step 8	Gel staining is weak	Staining time is too short or sample concentration is low	Extend staining time to 20 min or increase the sample concentration
	DNA sample stays inside wells and does not run into gels	The acrylamide concentration of the gel is too high	Try to run the sample using another gel with a lower acrylamide concentration
	Unexpected upper bands appear (low mobility)	Aggregations of the DNA nanostructures	Decrease the sample concentration or increase the running temperature to reduce aggregations
	Unexpected lower bands appear (high mobility)	Partially assembled structures appear or the structures are dissociated during electrophoresis	If the structures dissociate during the gel running, decrease the running temperature If partially assembled structures still exist, optimize the DNA folding strategy and sequences
42A(i), **Box 3**—step 12	Low recovery yield	The gel band from the sample was not completely crushed into small pieces	Cut the gel bands into small pieces, and crush completely before extraction
AFM characterization			
42B(i), **Box 4**—step 2	The density of DNA nanostructures (e.g. origami) is too high on the mica surface under AFM imaging	The deposit concentration of DNA nanostructures is high	Decrease the deposit concentration (approximately a few nanomoles for DNA origami), and do not add $NiCl_2$
	Very few DNA tiles are found on the mica surface	DNA tiles are not tightly bound to the mica surface; deposit concentration is low	Increase the deposit concentration of DNA tiles (~100–200 nM for DX tiles), and add $NiCl_2$ to enhance the binding

(continued)

TABLE 9 | Troubleshooting table (continued).

Step	Problem	Possible reasons	Solutions
TEM imaging			
42B(i), **Box 4**—step 15	The contrast of the TEM image (very dark) is too low to observe enzymes	Sample is overstained	Try to stain the sample for a shorter time, e.g., 5 s
	Background is too dirty under TEM	Uranyl formate precipitates from the solution	Prepare fresh uranyl formate solution
smTIRF characterization			
42C(x), **Box 5**—step 4	Cannot find focus on the slide surface	Laser focus is too far away from the field of view	Switch to a low-magnification objective (10× or 20×) to locate the laser focus and bring it to the center of the field of view using microscrews (*x* and *y* directions). Then switch back to the 60× objective
42C(x), **Box 5**—step 6	The fluorophores are quickly photobleached	There is dissolved oxygen in the solution photo-oxidizing the fluorophores	Add fresh OSS to the sample solution to remove the dissolved oxygen. Alternatively, double the OSS concentration

● **TIMING**

Steps 1–8, conjugation of DNA with enzymes and purification: 6–8 h
Steps 9 and 10, AE-FPLC purification of DNA-conjugated enzymes: 3–5 h
Steps 11–20, DNA –NAD^+ conjugation: ~4 h
Steps 21–26, HPLC purification of NAD^+-conjugated DNA: 1–2 d
Steps 27–39, denaturing PAGE purification of the oligonucleotides: 2 d
Step 40, assembly of DNA nanostructures (depending on the structures): 2–24 h
Step 41, assembly of enzymes onto DNA nanostructures: 2 h
Step 42, characterization of enzyme-assembled DNA nanostructures: 2–24 h
Step 43, purification of enzyme-assembled DNA nanostructures: 4–6 h
Step 44, Evaluation of enzyme activity: 2–13 h
Box 1, MALDI-TOF characterization of NAD^+-conjugated DNA: ~0.5 h
Box 2, native PAGE characterization of the enzyme assembly: 6–8 h
Box 3, AGE characterization and purification: 4–6 h
Box 4, AFM/TEM imaging of enzyme assembly: 1–2 h
Box 5, smTIRF characterization of DNA nanocaged enzymes: 4–6 h

ANTICIPATED RESULTS

Figure 1 demonstrates the main advantages of using DNA nanostructures to organize the spatial parameters and geometric arrangements of multienzyme systems on the nanoscale. As shown in **Figure 11a**, an enzyme cascade pair (e.g., GOx–HRP) can be organized by a rectangular DNA nanostructure with controlled distances between enzymes for probing the distance-dependent activity of the enzyme cascade reaction[25]. **Figure 11b** shows the assembly of biomimetic 'swinging arms', in which a single-stranded NAD^+-modified DNA molecule is used to channel the hydride transfer between two coupled dehydrogenases of G6PDH[86] and MDH[87] on a DX tile[28]. In **Figure 11c**, the activity of the G6PDH-MDH complex channeled by the NAD^+ swinging arm is ~90-fold higher than that obtained when using the same enzyme complex but with freely diffusing NAD^+ molecules. Further adjustments of the relative number and geometric arrangement of G6PDH and MDH produce an additional approximately twofold increase in activity for the G6PDH-NAD_2-MDH_2 structure and an additional approximately threefold increased activity for the G6PDH-NAD_4-MDH_4 structure. As shown in **Figure 11d**, a series of enzymes are encapsulated by DNA nanocages with >80% yield, and significantly enhanced turnover numbers are observed for most nanocaged enzymes[33]. In **Figure 11e**, a tweezers-like DNA nanodevice is designed to regulate the proximity between an enzyme (G6PDH) and a cofactor (NAD^+) for actuating the activity[29]. The switchable open and closed states of the tweezers are clearly visualized by AFM. Using this approach, several cycles of externally controlled enzyme inhibition and activation are successfully demonstrated (**Fig. 11f**).

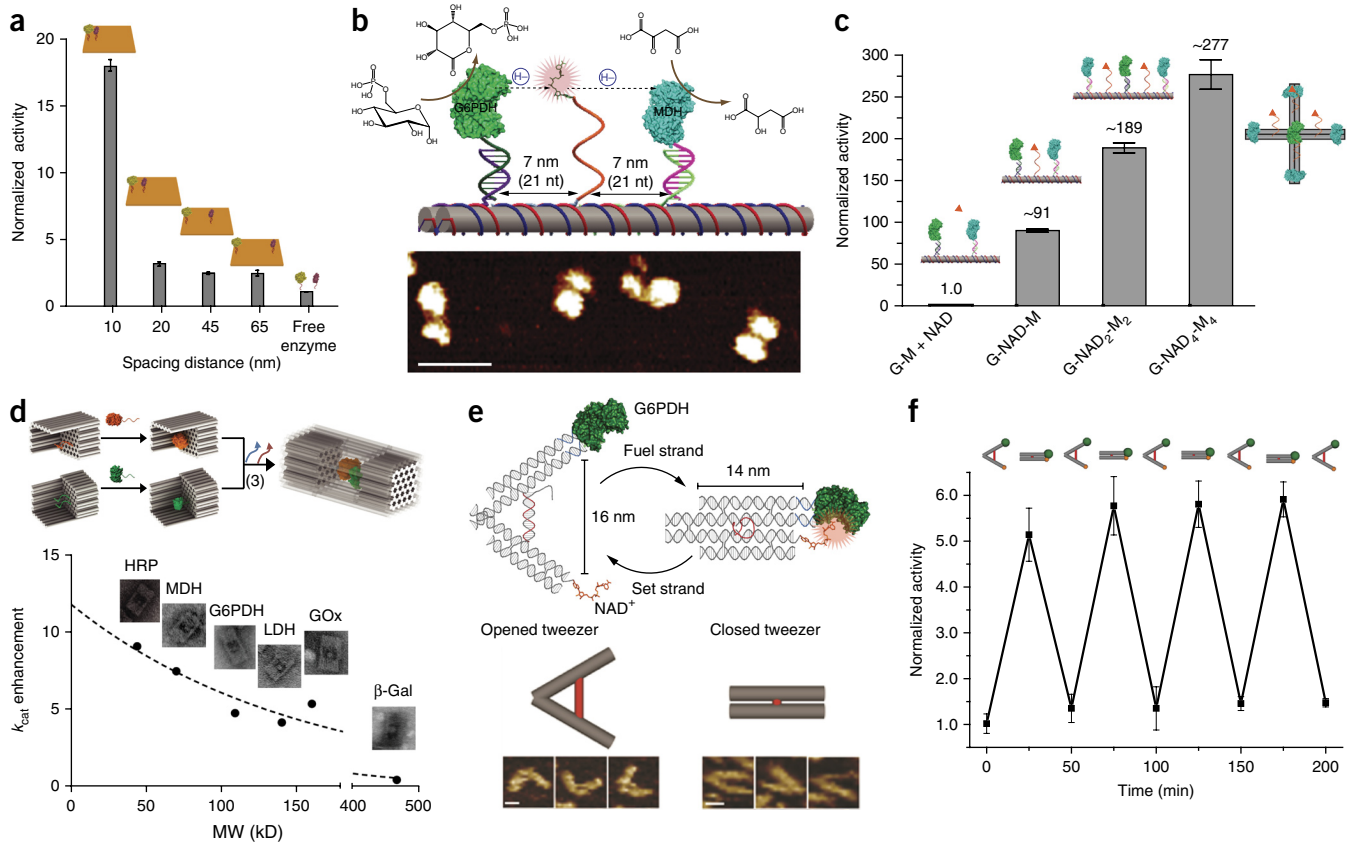

Figure 11 | The assembly of multienzyme complexes on DNA nanostructures. (**a**) Control for interenzyme distance for enzyme cascade reaction[25]. (**b**) A 'swinging arm'-channeled two-dehydrogenase complex (top) and AFM characterization (bottom) (ref. 28). Scale bar, 50 nm. (**c**) Activity of swinging arms can be enhanced adjusting the relative number of enzymes and their geometric arrangements: G6PDH-MDH assembly with free NAD^+ (G-M + NAD); G6PDH-NAD^+-MDH swinging-arm structure (G-NAD-M); G6PDH-NAD_2^+-MDH_2 swinging-arm structure (G-NAD_2-M_2) and G6PDH-NAD_4^+-MDH_4 structure (G-NAD_4-M_4). (**d**) The encapsulation of enzymes within a DNA nanocage and the enhanced turnover number of nanocaged enzymes depending on the size of encapsulated enzymes[33]. (**e**) A switchable set of DNA tweezers for regulating the distance between an enzyme (G6PDH) and a cofactor (NAD^+), and the AFM characterization of open and closed states of tweezers, scale bars, 10 nm. (**f**) Regulatory actuation of G6PDH/NAD^+ activity by switching tweezers between open and closed states. Error bars represent the standard deviation of three replicates ($N = 3$). β-Gal, β-galactosidase. **a** adapted with permission from ref. 25, American Chemical Society. **b** and **c** adapted with permission from ref. 28, Nature Publishing Group. **d** adapted with permission from ref. 33, Nature Publishing Group. **e** and **f** adapted with permission from ref. 29, Nature Publishing Group.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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