Preparation of aptamer-linked gold nanoparticle purple aggregates for colorimetric sensing of analytes

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Aptamers are single-stranded DNA or RNA molecules that can bind target molecules with high affinity and specificity. The conformation of an aptamer usually changes upon binding to its target analyte, and this property has been used in a wide variety of sensing applications, including detection based on fluorescence intensity, polarization, energy transfer, electrochemistry or color change. Colorimetric sensors are particularly important because they minimize or eliminate the necessity of using expensive and complicated instruments. Among the many colorimetric sensing strategies, metallic nanoparticle-based detection is desirable because of the high extinction coefficients and strong distance-dependent optical properties of the nanoparticles. Here, we describe a protocol for the preparation of aptamer-linked gold nanoparticle purple aggregates that undergo fast disassembly into red dispersed nanoparticles upon binding of target analytes. This method has proved to be generally applicable for colorimetric sensing of a broad range of analytes. The time range for the entire protocol is ~5 d, including synthesis and functionalization of nanoparticles, preparation of nanoparticle aggregates and sensing.

INTRODUCTION

Analogous to protein-based antibodies, aptamers are nucleic acid–based molecules that can be selected to bind essentially any molecule of choice[1,2]. Aptamers can be obtained by a combinatorial biology method called systematic evolution of ligands by exponential enrichment (SELEX)[1–4]. In a typical SELEX experiment, a target molecule is immobilized onto a column and a nucleic acid library containing 10^{14}–10^{15} random sequences is passed through the column. Those molecules binding strongly to the target are retained by the column, then eluted and amplified by polymerase chain reaction to seed the next round of selection. Detailed protocols for SELEX have been published in the literature and will not be covered here[5]. During the past 15 years the technologies used in the isolation of aptamers have been well developed and advanced. For example, automated selections can be achieved so that many selections with different targets can be performed in parallel[6]. With capillary electrophoresis as a separation tool, aptamers with desired affinities can be obtained in a single round of selection[7,8].

For full realization of aptamer-based sensing applications, the next challenge is to develop general methods to signal binding events[9–12]. The sensors should be easy to use, respond quickly, require no analytical instrumentation and be cost effective. To meet these challenges, investigators have reported several aptamer-based colorimetric sensors, such as those based on dye replacement[13] or nanoparticle assembly[14,15]. Recently, we reported a new class of colorimetric sensors based on target analyte-induced disassembly of nanoparticle aggregates[12]. It is an extension of our original design of colorimetric sensors based on DNAzyme-nanoparticle aggregates[16,17]. These sensors were shown to meet all the aforementioned criteria. Adenosine aptamer-linked aggregates are used here to illustrate the concept and provide experimental details. The same strategy has also been successfully applied to the construction of sensors responsive to cocaine.

The adenosine-sensitive nanoparticle aggregates consist of three components (**Fig. 1**): two DNA-functionalized nanoparticles (particles 1 and 2) and a linker DNA ($Linker_{Ade}$). The DNA for particle 1 is attached to a nanoparticle at its 3′ end. The linker DNA is designed so that the 5′ end, which is complementary to the DNA attached to particle 1, is separated from the adenosine aptamer at its 3′ end by a pentanucleotide sequence. The DNA for particle 2 is attached to a nanoparticle at its 5′ end and is complementary to the pentanucleotide and to the first portion of the adenosine aptamer. In the absence of adenosine or in the presence of other molecules such as other nucleosides, the aggregates are stable at room temperature and appear purple as a result of the surface plasmon effect[18]. In the presence of adenosine, however, the aptamer DNA (**Fig. 1** in green) switches its structure and binds two adenosine molecules[9,19]. As a result, only the pentanucleotide (**Fig. 1** in gray) in the linker DNA is left to bind particle 2. The five DNA base pairs are not strong enough to hold particle 2 at room temperature, leading to its dissociation and resulting in red individual gold nanoparticles. The most important element in designing such stimuli-responsive aggregates is the number of base pairs between the linker DNA and the DNA on particle 2, and the number of nucleotides from the aptamer sequence that are involved in binding to particle 2. The binding should be strong enough to hold the nanoparticles together but still allow the aptamer to bind target molecules and undergo rapid structure switching. Systematic studies on this topic have been reported by Li and co-workers[9,20].

An important feature of the design is that it is modular in nature. Simple replacement of the adenosine aptamer DNA sequence with those of other aptamers allows one to obtain sensors for a diverse range of analytes. The aptamers tested with this protocol have relatively low affinity for their targets (micromolar dissociation

constants), and those aptamers with higher affinity should also be effective. This protocol may not be applicable for analytes that have multiple aptamer binding sites (e.g., thrombin), because such analytes may favor assembly, rather than disassembly, of the nanoparticles[14,15]. The gold nanoparticles can also be replaced by other metallic nanoparticles, so that different color changes can be achieved in the presence of different analytes. In addition to metallic nanoparticles, other nanomaterials including magnetic nanoparticles, quantum dots, nanotubes or polymer beads can also be used to suit various applications.

TABLE 1 | DNA sequences and modifications.

DNA	Sequence (5′ to 3′)	Concentration
DNA1	TCACAGATGAGTAAAAAAAAAAAA-$(CH_2)_3$-SH	1 mM
DNA2	SH-$(CH_2)_6$-CCCAGGTTCTCT	1 mM
$Linker_{Ade}$	ACTCATCTGTGAAGAGAACCTGGGGGAGTATTGCGGAGGAAGGT	10 μM

MATERIALS

REAGENTS

- DNA1 and DNA2, desalted (1 mM) (**Table 1**; Integrated DNA Technologies Inc.)
- $Linker_{Ade}$, purified by polyacrylamide gel electrophoresis (10 μM) (**Table 1**; Integrated DNA Technologies Inc.)
- Hydrogen tetrachloroaurate(III) ($HAuCl_4$) solution (50 mM) (Aldrich)
- Trisodium citrate dihydrate (38.8 mM) (Aldrich)
- HCl, 36.5% (wt/wt) (Fisher)
- HNO_3, 65–70% (wt/wt) (Fisher)
- Tris-(2-carboxyethyl)phosphine hydrochloride (TCEP) (Sigma)
- Tris-(hydroxymethyl) aminomethane (Tris) (Aldrich)
- Glacial acetic acid (Fisher)
- Tris acetate buffer (500 mM, pH 8.2)
- Acetate buffer (a mixture of sodium acetate and acetic acid) (500 mM, pH 5.2)
- 300 mM NaCl, 25 mM Tris acetate, pH 8.2.
- 100 mM NaCl, 25 mM Tris acetate, pH 8.2
- Adenosine, cytidine, uridine, guanosine (50 mM each) (Aldrich)
- NaOH (12 M)

EQUIPMENT

- Two-neck flask (200 ml) with a condenser and a stopper
- Hot plate with magnetic stir bar
- Disposable scintillation vials (20 ml)
- Polypropylene microcentrifuge tubes (1.7 ml; catalog no. MCT-175-C; Axygen Scientific)
- Temperature-controlled UV-visible spectrophotometer (Hewlett-Packard 8453)
- Quartz UV-visible cell (Hellma)
- 0.2-μm syringe filter (Nalgene)
- Sep-Pak desalting column (Waters)

PROCEDURE

Preparation of gold nanoparticles ● TIMING 1 d

1| Prepare aqua regia by mixing 3:1 concentrated HCl:HNO_3 in a large beaker in a fume hood.

! CAUTION Be extremely careful when preparing and working with aqua regia. Wear goggles and gloves, and perform the experiment in a fume hood. Aqua regia should be freshly prepared and should never be stored in a closed vessel. The capped aqua regia bottle may explode. Render it safe by dilution and neutralization.

2| Soak the 200 ml two-neck flask, magnetic stir bar, stopper and condenser in aqua regia for at least 15 min. Rinse the glassware with copious amount of deionized water and then Millipore-filtered water[21].

▲ CRITICAL STEP Obtaining high-quality nanoparticles is the first important step towards the success of the experiment. Care should be taken to make sure that no contamination is introduced during nanoparticle synthesis.

3| Load 98 ml of Millipore water into the two-neck flask. Add 2 ml of 50 mM $HAuCl_4$ solution so that the final $HAuCl_4$ concentration is 1 mM.

4| Connect the condenser to one neck of the flask, and place the stopper in the other neck. Put the flask on the hot plate to reflux while stirring.

5| When the solution begins to reflux, remove the stopper. Quickly add 10 ml of 38.8 mM sodium citrate, and replace the stopper. The color should change from pale yellow to deep red in 1 min. Allow the system to reflux for another 20 min.

6| Turn off heating and allow the system to cool to room temperature (23–25 °C) under stirring. The diameter of such prepared nanoparticles is ~13 nm. The extinction value of the 520-nm plasmon peak is ~2.4, and the nanoparticle concentration is ~13 nM. The color should be burgundy red, and the nanoparticle shape should be spherical under transmission electron microscopy (TEM). (Note: Nanoparticles of 13 nm diameter are used because they can be synthesized in high quality (20% coefficient of size variation) with high reproducibility[22], and the protocol of functionalization with DNA has been well established[21]. Nanoparticles of other sizes should also be effective. For example, we tested 40-nm-diameter nanoparticles and observed similar adenosine-induced color changes (our unpublished results).)

? TROUBLESHOOTING

■ **PAUSE POINT** The prepared nanoparticles are stable for months when stored in a clean container (glass or plastic) at room temperature. Do not freeze the nanoparticles.

Functionalization of nanoparticles with thiol-modified DNA ● **TIMING 2–3 d**

7| Soak two disposable scintillation vials (20 ml volume) in 12 M NaOH for 1 h at room temperature. Rinse the vials with copious amounts of deionized water and then Millipore water.

! CAUTION Be extremely careful when preparing and working with concentrated NaOH. Wear goggles and gloves, and perform the experiment in a fume hood. When preparing 12 M NaOH solution, the temperature of the system increases significantly. The concentrated NaOH solution can be reused many times to soak glass vials.

▲ **CRITICAL STEP** If the glass vials are not treated with concentrated NaOH, nanoparticles tend to stick to the surface of the vials, especially after addition of NaCl to the particles. If this occurs, the effective concentration of nanoparticles decreases.

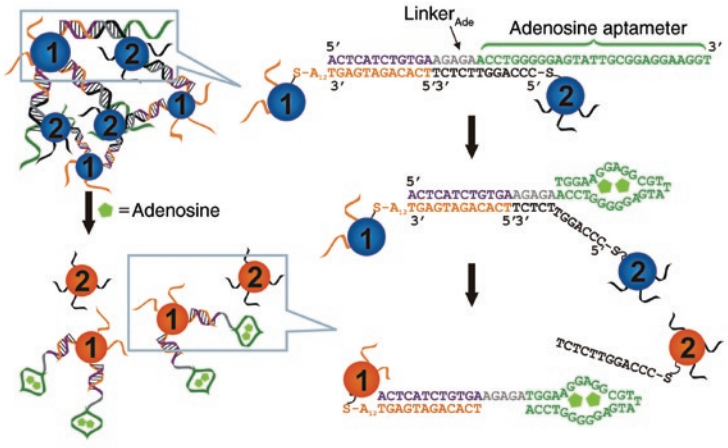

Figure 1 | Adenosine-induced disassembly of nanoparticle aggregates for colorimetric detection of adenosine. Nanoparticles 1 and 2 are functionalized with two different DNA molecules through thiol-gold chemistry. The two kinds of nanoparticles are linked by Linker$_{Ade}$ to form aggregates. In the presence of adenosine, the aggregates disassemble to give dispersed red nanoparticles.

8| Prepare 10 mM TCEP.

▲ **CRITICAL STEP** TCEP should be freshly prepared.

9| Pipette 9 μl of 1 mM DNA1 into a microcentrifuge tube and 9 μl of 1 mM DNA2 into another one.

10| Add 1 μl of 500 mM acetate buffer (pH 5.2) and 1.5 μl of 10 mM TCEP to each tube to activate the thiol-modified DNA. Incubate the sample at room temperature for 1 h.

11| Transfer 3 ml of the already prepared gold nanoparticles to each of the two NaOH-treated glass vials, and then add the TCEP-treated thiol DNA with gentle shaking by hand.

12| Cap the two vials and store them in a drawer at room temperature for at least 16 h. Magnetic stirring may also be applied to facilitate the reaction. (Although all the operations described in this protocol can be carried out under light, it is advised to keep nanoparticles in the dark for long-term storage.)

13| Add 30 μl of 500 mM Tris acetate (pH 8.2) buffer dropwise to each vial with gentle hand shaking. The final Tris acetate concentration is 5 mM.

14| Add 300 μl of 1 M NaCl dropwise to each vial with gentle hand shaking. Store the two vials in a drawer for at least another day before use. These two types of functionalized gold nanoparticles correspond to particles 1 and 2 in **Figure 1**.

■ **PAUSE POINT** The functionalized nanoparticles can still be used to form aggregates even after stored at room temperature for months, although their properties may change slightly with the passage of time as a result of events such as degradation of DNA. These changes could affect the properties of the prepared aggregates. To be sure that the results are consistent, use freshly functionalized nanoparticles.

Preparation of aptamer-linked nanoparticle aggregates ● **TIMING** 1 d

15| Transfer 500 μl of functionalized particles 1 and 2 into two 1.7-ml microcentrifuge tubes, respectively.

16| Centrifuge the two tubes at 16,110*g* at room temperature (23–25 °C) on a benchtop centrifuge for 15 min.

17| Remove the two tubes from the centrifuge. The supernatant should be clear, and the nanoparticles should be at the bottom of the tubes. If a red color can still be observed in the supernatant, centrifuge for another 5 min.

18| Gently pipette off as much supernatant as possible to remove free DNA. Again, disperse the nanoparticles in 200 µl of buffer containing 100 mM NaCl, 25 mM Tris acetate, pH 8.2.
? TROUBLESHOOTING

19| Centrifuge again for 10 min at 16,110*g* at room temperature (23–25 °C).

20| Remove the supernatant. Again, disperse the nanoparticles in 500 µl of buffer containing 300 mM NaCl, 25 mM Tris acetate, pH 8.2. We found that most of the free DNA can be removed by two centrifugations. If desired, repeat Steps 16–19 to remove more free DNA.

21| Mix the two nanoparticle solutions.

22| Mix 10 µl of 10 µM $Linker_{Ade}$ DNA with the nanoparticles so that its final concentration is 100 nM.

23| Incubate the nanoparticles at 4 °C for at least 1 h. The solution color should change from red to purple. To obtain nanoparticle aggregates in high yield, incubate the solution longer. (We found that after overnight incubation, almost all of the nanoparticles went into aggregates because no red color was observable in the supernatant. After long-term incubation, the aggregates may grow large enough to precipitate out of solution. These large aggregates, however, can still be used for sensing applications. With brief agitation by a pipette, the aggregates can be resuspended.)
? TROUBLESHOOTING
■ **PAUSE POINT** The aggregates can be stored at 4 °C for weeks and still maintain their sensing activity.

Optimizing melting conditions so that reaction will proceed at room temperature ● **TIMING** 1 d
24| Centrifuge the aggregates at 800*g* for 1 min at room temperature (23–25 °C).

25| Remove the supernatant. Redisperse the aggregates in 500 µl of buffer containing 300 mM NaCl, 25 mM Tris acetate, pH 8.2.

26| Take 50 µl of the just-prepared aggregates and dilute to 200 µl with buffer. The dilution buffer contains 25 mM Tris acetate, pH 8.2 and 100 mM NaCl. After dilution, the final NaCl concentration is 150 mM. The drop in NaCl concentration from 300 mM to 150 mM does not cause significant changes to the optical properties of the nanoparticle aggregates (only a slight increase in the extinction ratio was observed), because the melting temperature of the aggregates in 150 mM NaCl is still much higher than room temperature.

27| Transfer the diluted aggregates into a UV-visible cell; seal the cell with Parafilm.

28| Place the sealed cell in the temperature-controlled UV-visible sampling chamber, and measure the extinction spectra for a range of temperatures (e.g., from 15 °C to 60 °C at intervals of 2 °C). Allow at least 1 min for equilibration after reaching each designated temperature. Agitate the cell before taking each measurement to make sure that the aggregates are suspended homogeneously.

29| Record and plot the extinction at 260 nm versus temperature. Initially, the extinction may be constant or decrease slightly with increasing temperature. After reaching a certain temperature, the extinction begins to increase sharply.

30| Record the temperature at which the extinction starts to increase. The optimal temperature for detection is ~2–3 °C below this.

31| Repeat Steps 27–30 at different NaCl concentrations. Usually the optimal temperature increases with increasing NaCl concentration. Because experiments are the most convenient to conduct at room temperature, adjust the NaCl concentration to maintain the optimal temperature around room temperature. A good starting point for freshly functionalized nanoparticles is 150 mM NaCl.
? TROUBLESHOOTING
▲ **CRITICAL STEP** The nanoparticles are linked by DNA base-pairing interactions. A certain concentration of NaCl is required to stabilize the nanoparticle aggregates. The higher the salt concentration, the more stable the aggregates. The stability of nanoparticle aggregates can be evaluated by melting studies. Aptamer binding to its target analyte, on the other hand, is less dependent on NaCl. We find that doubling the NaCl concentration under optimized conditions can inhibit the color change.

Detection of analytes with the nanoparticle aggregates ● **TIMING** 1 d

32| Add 1 µl of 50 mM adenosine or any other nucleoside solution to 49 µl of nanoparticle aggregates (final concentration 1mM) with optimized NaCl concentration to observe color change. To completely dissolve 50 mM adenosine or guanosine, heat the solutions in a boiling water bath.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

Table 2 | Troubleshooting table.

STEP NUMBER	PROBLEM	SOLUTION
Step 6	The color of nanoparticles appears purple, and the shape of nanoparticles under TEM is not spherical.	Be sure to wash the glassware well with aqua regia. The quality of nanoparticles is strongly affected by the cleanness of glassware[22]. It is recommended that a set of glassware be reserved for nanoparticle synthesis (no contamination from other organic synthesis). Filter all solutions with a 0.2-µm syringe filter, including $HAuCl_4$ and trisodium citrate. Stirring should be vigorous and the addition of citrate should be fast.
Step 18	After centrifugation, nanoparticles cannot be redispersed, and only large aggregates are observed.	This is probably due to poor functionalization of the nanoparticles. Confirm DNA concentration by UV-visible spectrophotometry. Check DNA purity and the presence of the thiol group by gel electrophoresis and by mass spectrometry, respectively. Purify by gel electrophoresis if necessary. If the problem persists, increase TCEP concentration 10 times and pass the mixture through a Sep-Pak desalting column to remove excess TCEP. Another possibility is the presence of self-complementary sequences in the DNA attached to the nanoparticles, so that the nanoparticles can aggregate by DNA-pairing interactions even without a separated linker DNA. To confirm this possibility, add water instead of buffer to the aggregates. Recovery of red color confirms nanoparticle self-aggregation. To avoid such self-complementary sequences, use DNA secondary structure prediction programs such as Mfold for sequence optimization[23].
Step 23	No purple aggregates form and nanoparticles are still in a dispersed state.	We find that the source of microcentrifuge tubes can have a strong effect on the aggregation of nanoparticles. We find that polypropylene tubes from Axygen Scientific (see Equipment) can give high aggregate yield. Check the DNA sequences and make sure that these are correct. Repeat the experiment with 25 mM Tris acetate buffer, pH 8.2 with 500 mM NaCl for incubation. If the particles still do not aggregate, functionalize fresh particles. Try facilitating aggregation by warming the sample to 70 °C for 1 min and then slowly cool the sample in a water bath to room temperature over 2 h. If this problem persists, run a non-denaturing gel to observe the complex between DNA1/$Linker_{Ade}$, DNA2/$Linker_{Ade}$ and DNA1/DNA2/$Linker_{Ade}$. If the DNA sequences are correct, the complexes should migrate differently than the single-stranded DNA.
Step 31	The aggregates do not melt, even at very high temperatures (i.e., in boiling water).	Centrifuge the aggregates (in 300 mM NaCl) gently and remove most of the supernatant to make concentrated aggregates. Add 25 mM Tris acetate, pH 8.2 buffer (no NaCl) to dilute the aggregates 20 times to a final concentration of 15 mM NaCl. If the color of the aggregates changes to red at room temperature, repeat the melting study at a lower NaCl concentration. Failure of the aggregates to turn red at the low NaCl concentration could be due to poor functionalization of the nanoparticles. See troubleshooting for Step 18.
Step 32	No color change is observed with 1 mM (final concentration) added adenosine.	Make sure NaCl concentration has been adjusted so that the optimal temperature (see Step 30 for identification of the optimal temperature for detection) is around room temperature. If this is confirmed and the problem persists, run a non-denaturing gel with DNA2 and $Linker_{Ade}$ in the presence and absence of 5 mM adenosine. The two lanes should migrate differently if the aptamer part can bind adenosine. Because the solubility of adenosine at room temperature is limited, but high in boiling water, confirm the concentration of adenosine.

ANTICIPATED RESULTS

Upon addition of adenosine, the color of the sensor solution should change from purple to red. Addition of other nucleosides should not change the color of the solution. Under optimized conditions (such as NaCl concentration and temperature), the color change should be instantaneous. Shown in **Figure 2a** are the typical UV-visible spectra of nanoparticles in the dispersed (red curve) and aggregated states (blue curve). Upon disassembly, the extinction at the 522-nm plasmon peak increases while the extinction in the 700-nm region decreases. Therefore, the ratio of extinction at 522 nm to that at 700 nm can be used to quantify the assembly state and color of nanoparticles, with a high ratio indicating dispersed nanoparticles of red color. A typical TEM image of an aggregate is shown in **Figure 2b**. Thousands of nanoparticles are linked by DNA to form a rigid network structure. The kinetics of sensor color change in the presence of 1 mM adenosine, cytidine, uridine or guanosine is presented in **Figure 1c**, and a photograph of the samples is shown as an inset. Only the sample with added adenosine showed a rapid color change from purple to red, whereas the other three samples remained purple. The kinetics of color change also depends on the concentration of adenosine added, which can be used for quantitative analysis (**Fig. 1d**).

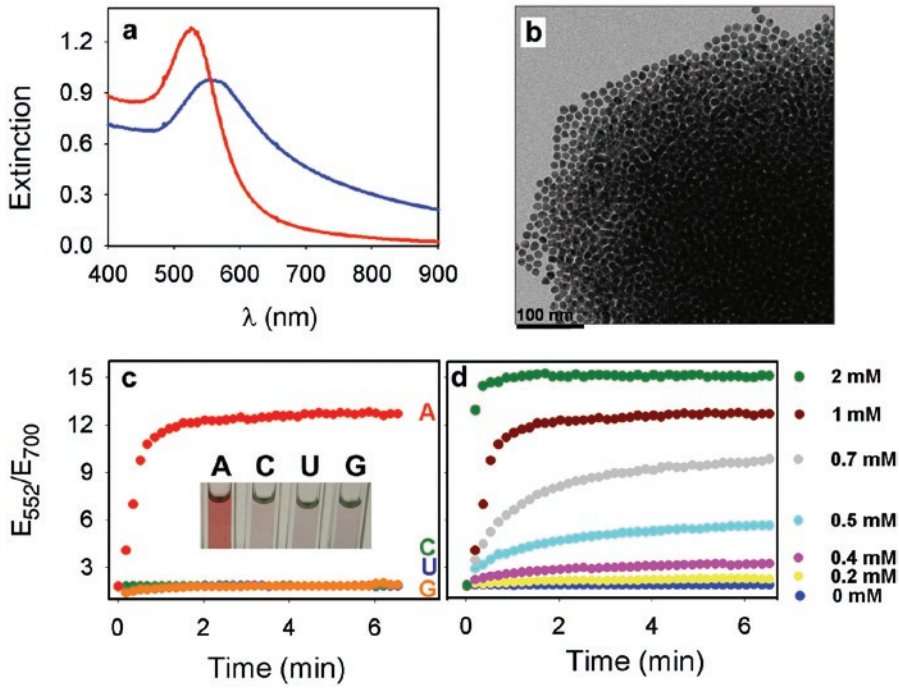

Figure 2 | Colorimetric detection of adenosine with aptamer-assembled nanoparticle aggregates. (**a**) UV-visible spectra of dispersed (red) and aggregated (blue) gold nanoparticles. (**b**) TEM of aptamer-linked gold nanoparticle aggregates. The scale bar is 100 nm. (**c**) Kinetics of color change of adenosine aptamer-assembled aggregates in the presence of 1 mM nucleosides. Inset: photograph of the four samples with designated nucleoside added. (**d**) Kinetics of color change of the aggregates with varying adenosine concentrations.

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COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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