A DNA nanomachine that maps spatial and temporal pH changes inside living cells

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DNA nanomachines are synthetic assemblies that switch between defined molecular conformations upon stimulation by external triggers. Previously, the performance of DNA devices has been limited to in vitro applications. Here we report the construction of a DNA nanomachine called the I-switch, which is triggered by protons and functions as a pH sensor based on fluorescence resonance energy transfer (FRET) inside living cells. It is an efficient reporter of pH from pH 5.5 to 6.8, with a high dynamic range between pH 5.8 and 7. To demonstrate its ability to function inside living cells we use the I-switch to map spatial and temporal pH changes associated with endosome maturation. The performance of our DNA nanodevices inside living systems illustrates the potential of DNA scaffolds responsive to more complex triggers in sensing, diagnostics and targeted therapies in living systems.

DNA provides remarkable specificity and versatility in the field of molecular recognition and therefore comprises an excellent nanoscale building-block for the creation of precisely self-assembled nanostructures. DNA nanomachines are artificially designed assemblies that change their states in response to inputs such as chemical stimuli generated by environmental cues, external triggers. Previously, the performance of DNA devices has been limited to in vitro applications. Here we report the construction of a DNA nanomachine called the I-switch, which is triggered by protons and functions as a pH sensor based on fluorescence resonance energy transfer (FRET) inside living cells. It is an efficient reporter of pH from pH 5.5 to 6.8, with a high dynamic range between pH 5.8 and 7. To demonstrate its ability to function inside living cells we use the I-switch to map spatial and temporal pH changes associated with endosome maturation. The performance of our DNA nanodevices inside living systems illustrates the potential of DNA scaffolds responsive to more complex triggers in sensing, diagnostics and targeted therapies in living systems.

Design and working principle of the I-switch

The I-switch consists of three oligonucleotides O1, O2 and O3, where O1 and O2 are hybridized onto sites adjacent to O3, leaving a one-base gap as shown in Fig. 1a. O1 and O2 have single-stranded cytosine-rich overhangs (see Supplementary Fig. S1) designed such that each overhang forms one-half of a bimolecular i-motif. At acidic pH, these overhangs are protonated and the assembly can fold to form an intramolecular i-motif (Fig. 1a).

In vitro characterization of the I-switch

Circular dichroism (CD) spectroscopy on the I-switch shows that this assembly adopts an extended conformation at pH 7.3, whereas at pH 5 the single-stranded overhangs form an i-motif, yielding a ‘closed state’ (see Supplementary Fig. S2). The I-switch, fluorescently labelled at its 3’ and 5’ termini with Alexa-488 and Alexa-647 on O1 and O2 respectively, shows fluorescence resonance energy transfer (FRET) at pH 5, with a transfer efficiency of 54–60% consistent with an interfluorophore distance of 4.6 ± 0.5 nm. This is in agreement with the theoretical estimate of 4 nm for the end-to-end distance in such an i-motif. No significant FRET was observed in the open state at pH 7.3 (see Supplementary Fig. S3). FRET was further confirmed through time-resolved fluorescence measurements (Fig. 1d, inset) of the donor in the closed and open states. At pH 7.3, donor lifetimes of the Alexa-488-labelled I-switch showed only a marginal difference between the donor-only-labelled I-switch (~3.77 ns) and the doubly-labelled (Alexa-488/Alexa-647) complex (~3.50 ns). However, at pH 5, the donor lifetime showed a significant change to 2.1 ns for the doubly-labelled I-switch, with the donor-only I-switch showing only a slight decrease (~3.02 ns) (see Supplementary Fig. S4). To see if the I-switch could switch reversibly between the ‘closed’ and ‘open’ states upon variation of pH, 5 nM of doubly-labelled I-switch (Alexa-546/647 FRET pair) in 100 mM KCl was subjected to pH cycling between pH 5 and 8 by adding acid or base to the
cargo functions such as sorting of secretory molecules, growth factors, nutrients and toxins (see Supplementary Fig. S6 for a schematic). As early endosomes mature to late endosomes and finally to lysosomes, they undergo a characteristic change of pH ranging from 6 to 6.2 in early endosomes, to pH 5.5 in late endosomes and pH 5 in lysosomes. We therefore investigated the capacity of the I-switch to function inside living cells by investigating endosome maturation in Drosophila haemocytes. Drosophila haemocytes were ‘pulsed’ or incubated with a mixture of I-switch labelled with Bodipy TMR (80 nM) and fluorescein isothiocyanate (FITC)-conjugated dextran (FITC-dextran) (1 mg ml\(^{-1}\)).

Figure 2c shows an image of Drosophila haemocytes pulsed (5 min) and chased for 5 min, fixed and then imaged in a confocal microscope. Importantly, the I-switch was found to be localized in distinct punctate structures ~1 μm in size. When these images were overlayed with co-internalized FITC-dextran images, these puncta were found to co-localize (~90%), indicating that the I-switch indeed entered endosomes (Fig. 2c). To check the integrity of the I-switch in living cells over the time period required for endosomal maturation, we next followed the I-switch inside endosomes for periods of 5 min, 1 h and 2 h. Up to 2 h, the donor and acceptor labels of the I-switch remain co-localized in endosomes, indicating the integrity of the I-switch (see Supplementary Fig. S7a). This observation was further confirmed by fluorescence lifetime experiments inside endosomes, demonstrating that the fluorophores on the I-switch are within the pH 5 and 7.3.
known that cargo entering cells by means of endocytic pathways such as the ALBR pathway fuse with FITC–dextran-labelled endosomes at the indicated chase times such as the ALBR pathway fuse with FITC–dextran-labelled endosomes. We have demonstrated proof of concept by marking the temporal pH changes for a given protein. We have developed a strategy to tag it to any given biotinylated protein. We have demonstrated proof of concept by marking the receptor-mediated endocytic (RME) pathway of transferrin in Drosophila SR+ cells by tagging the I-switch to transferrin (Fig. 4a). Biotinylated I-switch (I(B)) was first conjugated to streptavidin (SA), which was subsequently conjugated to biotinylated transferrin (Tf(B)) to yield transferrin-modified I-switch (I(B,SA–Tf(B)).

**Tracking pH changes for a given protein**

To make the I-switch valid as a FRET-based sensor of spatiotemporal pH changes in the environment of a protein of interest, we have developed a strategy to tag it to any given biotinylated protein. We have demonstrated proof of concept by marking the receptor-mediated endocytic (RME) pathway of transferrin in Drosophila SR+ cells by tagging the I-switch to transferrin (Fig. 4a). Biotinylated I-switch (I(B)) was first conjugated to streptavidin (SA), which was subsequently conjugated to biotinylated transferrin (Tf(B)) to yield transferrin-modified I-switch (I(B,SA–Tf(B)).
Figure 3 | Spatial and temporal mapping of pH changes during endocytosis using the I-switch in living cells. a, Pseudocolour D/A map of haemocytes pulsed with I-switch (Alexa-488/647) at the indicated chase times. Scale bar, 5 μm. b, Histograms showing D/A ratios of ~80 individual endosomes after chase times of 5 min, 1 h and 2 h. c, The table summarizes endosomal pH variation as a function of time. The observed D/A values were converted to their corresponding pH values from the intracellular calibration curve in Fig. 2a. d, Real-time monitoring of the rate of acidification during endocytosis. Cells were pulsed with I-switch, washed, then imaged over 2 h. Five distinct cells were imaged for each time point and mean D/A of two experiments was plotted with time. Error bars represent the standard error of the mean. Scale bar, 5 μm.

(Fig. 4a) characterized by gel electrophoresis and size exclusion chromatography (Fig. 4a; see also Supplementary Figs S9 and S10).

Drosophila SR⁺ cells were incubated with I<sub>B-SA-Tf<sub>B</sub></sub> (Alexa-488/647-labelled I<sub>B</sub>) on ice for 15 min, chased for 10 min, the surface-bound probe was then stripped using a low-pH stripping buffer, fixed and then imaged. Overlaying donor and acceptor images show co-localization in discrete punctate structures (Fig. 4b). These were completely absent in control cells pulsed with the I-switch lacking Tf<sub>B</sub>. This was further confirmed by competition experiments with unlabelled transferrin (see Supplementary Fig. S11). Transferrin marks recycling endosomes that are comparatively less acidic than the late endosomes and lysosomes. Figure 4c, d shows FRET maps of recycling endosomes marked with I<sub>B-SA-Tf<sub>B</sub></sub> at t = 20 min before (Fig. 4c) and after (Fig. 4d) addition of nigericin. The fold increase of D/A value (blue, pH 5, to red, pH 7) (Fig. 4d) was used to quantify the acidity in these organelles. Before quantification, a pH calibration curve of I<sub>B-SA-Tf<sub>B</sub></sub> was generated in this cell line as described earlier; this was shown to overlap with the in vitro pH curve (Fig. 4g) indicating that the pH sensing capability of the I-switch is unchanged after conjugation. When the D/A values of each endosome (n ~ 50) were quantified and compared with the standard curve, it revealed a mean pH of 6.3 ± 0.1 (Fig. 4g, black square) which is consistent with the mildly acidic nature of recycling endosomes. This reveals that the I-switch is a high-performance reporter that, through protein conjugation, is able to measure the pH of its environment.

Conclusions
We have demonstrated the successful operation of an artificially designed DNA nanomachine inside living cells, and have shown that these nanomachines work as efficiently inside cells as in vitro. Importantly, because the byproducts of a complete cycle for the I-switch are nontoxic, being water and salt, they do not hamper its processivity. The I-switch has a pH sensitivity between pH 5.5 and 6.8 and offers complementary information to that obtained through the use of small-molecule fluorescent pH probes<sup>27</sup>. Unlike such pH probes, the I-switch is a FRET-based sensor that is equally bright at both physiological and acidic values of pH. It is also photostable and is a ratiometric probe. Most importantly, with pH probes based on green fluorescent proteins (GFPs)<sup>22</sup> or small molecules one is limited by a fixed wavelength, but the I-switch, which is an artificially designed DNA scaffold, can incorporate any appropriate FRET pair. It can therefore be used to simultaneously follow multiple proteins, with each protein bearing a distinct FRET pair, thus defining it as a powerful probe to study compartment mixing in intracellular sorting or trafficking events. The response of the I-switch is on timescales of 1–2 min, allowing it to be used as a reporter of fine spatial and temporal pH changes associated with biological processes that occur on longer timescales, such as pH variations associated with viral infections<sup>29</sup>, phagocytosis<sup>30</sup>, chemotaxis<sup>31</sup>, apoptosis<sup>32</sup> and defective acidification in tumour cells<sup>33</sup>.

This first generation of DNA nanoswitches, although promising, must overcome some limitations before they can be as widely used as current pH sensors. Faster DNA switches that can measure pH changes on shorter timescales, as well as over different ranges of pH sensitivity, need to be engineered. Most importantly, to address compartment mixing issues, methods to specifically target such DNA nanostructures to different cellular organelles would be a crucial development. It is notable that, in analogy to cellular machines, artificially designed DNA nanomachines are responsive to a molecular cue, can be specifically coupled to the cellular environment, and yet function independently within the crowded cellular milieu. Thus, DNA nanomachine function on the nanoscale can be efficiently transduced to cellular length scales. The robustness and level of performance of DNA nanomachines open up a...
wide range of possibilities for DNA-based cellular devices for sensing, diagnostics and targeted therapies in living systems.

Methods

Materials. DNA oligonucleotides purified by high-performance liquid chromatography (O1, O2 and O3) were purchased from MWG Biotech. All CD scans were performed on a JASCO J-815 spectrophotometer equipped with a temperature controller at 1 μM strand concentration in the appropriate buffer. Spectra are presented as an average of five successive scans. For fluorescence, samples were excited at 488 nm and emission was collected between 505 nm and 750 nm on a Fluorolog-Spex or a JASCO J-815 spectrophotometer. The FRET efficiencies were calculated using the formula

\[ \text{FRET} = 1 - \frac{I_{\text{B-SA}}}{I_{\text{B}}/I_{\text{D}}} = 1 + (R/R_p)^m \]  

where \( I_{\text{B-SA}} \) and \( I_{\text{B}} \) are the fluorescence intensities of the donor and acceptor, respectively, and \( R \) and \( R_p \) are the Förster radius of the donor-acceptor pair and the radius of the donor-acceptor pair at the point of Förster resonance, respectively.

Cell culture, protein conjugation and imaging. Haemocytes were obtained from wandering third instar Drosophila larvae as described previously. For pH measurement experiments, cells were imaged live, after chasing the probes for the stated timepoints. For co-localization of Rab-5 with the I-switch, transgenic flies expressing Rab-5–GFP were crossed to flies expressing Heme–Gal4, and GFP-Positive progeny were used for haemocyte cell culture. Stable lines of flies expressing Rab-5–GFP were crossed to flies expressing Heme–Gal4, and GFP-positive progeny were used for haemocyte cell culture. Stable lines of Drosophila SR⁺ cells expressing the human transferrin receptor (Gupta et al., unpublished) were used for labelling experiments. Wide-field and confocal images were collected using a Nikon inverted microscope and an Olympus Fluoview 1000 confocal microscope respectively. Donor and acceptor images were overlayed and endosomes showing co-localization were further quantified using ImageJ. Five independent measurements were presented as the mean ± standard error (s.e.m.). \( I_{\text{B-SA}} \) comprising O1, O2 and O3-biotinylated O3 was conjugated to SA in a 1:1 ratio. \( I_{\text{B-SA}} \) was conjugated to TfB similarly in an \( I_{\text{B-SA}}/TfB \) ratio of 1:2. For details see Supplementary Information.

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References

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Author contributions

S.M., S.M.G. and Y.K. conceived, designed and analysed the experiments. S.M. performed time-resolved experiments. G.D.G. performed the experiments. S.M. and S.M.G. wrote the paper. D.G. performed the experiments. Correspondence and requests for materials should be addressed to Y.K.

Additional information

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