

# Cyanine fluorophore derivatives with enhanced photostability

Roger B Altman<sup>1</sup>, Daniel S Terry<sup>2,7</sup>, Zhou Zhou<sup>1,7</sup>,  
Qinsi Zheng<sup>3</sup>, Peter Geggier<sup>4</sup>, Rachel A Kolster<sup>4</sup>,  
Yongfang Zhao<sup>4</sup>, Jonathan A Javitch<sup>4,5</sup>,  
J David Warren<sup>6</sup> & Scott C Blanchard<sup>1-3,6</sup>

Fluorescence applications requiring high photostability often depend on the use of solution additives to enhance fluorophore performance. Here we demonstrate that the direct or proximal conjugation of cyclooctatetraene (COT), 4-nitrobenzyl alcohol (NBA) or Trolox to the cyanine fluorophore Cy5 dramatically enhanced fluorophore photostability without otherwise affecting its native spectral characteristics. Such conjugation is a powerful means of improving the robustness of fluorescence-based applications demanding long-lived, nonblinking fluorescence emission.

## BRIEF COMMUNICATIONS

### Cyanine fluorophore derivatives with enhanced photostability

Roger B Altman<sup>1</sup>, Daniel S Terry<sup>2,7</sup>, Zhou Zhou<sup>1,7</sup>,  
Qinsi Zheng<sup>3</sup>, Peter Geggier<sup>4</sup>, Rachel A Kolster<sup>4</sup>,  
Yongfang Zhao<sup>4</sup>, Jonathan A Javitch<sup>4,5</sup>,  
J David Warren<sup>6</sup> & Scott C Blanchard<sup>1-3,6</sup>

Fluorescence applications requiring high photostability often depend on the use of solution additives to enhance fluorophore performance. Here we demonstrate that the direct or proximal conjugation of cyclooctatetraene (COT), 4-nitrobenzyl alcohol (NBA) or Trolox to the cyanine fluorophore Cy5 dramatically enhanced fluorophore photostability without otherwise affecting its native spectral characteristics. Such conjugation is a powerful means of improving the robustness of fluorescence-based applications demanding long-lived, nonblinking fluorescence emission.

Small organic fluorophores are powerful research tools in biological imaging that have enabled important insights into both cellular and molecular processes. However, their performance can be compromised by undesirable photophysical properties that limit both the fluorescence quantum yield and the total number of photons emitted before photobleaching. Such issues include both transient (blinking) and irreversible (photobleaching) light-induced transitions to dark states. Dark state transitions are particularly limiting in single-molecule studies that demand high illumination intensities. These problems are especially common in longer-wavelength fluorophores, such as Cy5, widely used in fluorescence resonance energy transfer-based investigations and applications demanding high signal-to-noise ratios.

The addition of small-molecule solution additives is a powerful means of minimizing fluorophore blinking and photobleaching both *in vivo* and *in vitro*<sup>1-5</sup>. Common additives include triplet state quenchers (TSQs) such as cyclooctatetraene (COT), 4-nitrobenzyl alcohol (NBA) and *tert*-butylhydroquinone-2-carboxylic acid (Trolox)<sup>6,7,8</sup> that act as a concentration-dependent means to reduce blinking rates, dark-state lifetimes and photobleaching rates<sup>9</sup>.

Despite their advantages, TSQs have key limitations, including poor aqueous solubility, problems with membrane permeability and biological toxicity. To circumvent these issues, here we show that direct or proximal linkage of TSQs to the Cy5 fluorophore reduced blinking and photobleaching in both deoxygenated and oxygenated environments to extents exceeding those using TSQs in solution. We also observed enhanced Cy5 performance in living cells, suggesting these new fluorophore derivatives may be valuable for *in vivo* applications<sup>10</sup>.

*In vivo* single-molecule studies demonstrating that TSQs operate as a concentration-dependent means to affect the photophysical properties of cyanine fluorophores suggest a collision-based mode of action<sup>9</sup>. To determine whether additional enhancements to fluorophore performance could be achieved by increasing the effective TSQ concentration beyond the solubility limit while simultaneously bypassing issues related to toxicity, we synthesized specific Cy5-TSQ conjugates in which we directly linked COT, NBA or Trolox to the fluorophore through a flexible, 12-atom linker (Supplementary Fig. 1). We developed a general strategy for the synthesis of such compounds by first modifying each TSQ to contain a single, unique functional group followed by coupling this to the common dye available, bis-reactive N-hydroxysuccinimide ester (NHSE)-Cy5 fluorophore to yield a mono-functionalized NHSE-Cy5-TSQ species at high efficiency (>38–69%) and purity (>99%) (Fig. 1a and Supplementary Notes).

Bulk fluorescence measurements of the TSQ-conjugated Cy5 fluorophores demonstrated that absorption and emission spectra of TSQ-fluorophore conjugates were largely indistinguishable from those of the parent Cy5 compound, aside from modest shifts in fluorescence quantum yield (Supplementary Fig. 2a,b). The quantum yields for Cy5-COT, Cy5-NBA and Cy5-Trolox were increased by 20%, unchanged or decreased by 20%, respectively, compared to that for Cy5. Such changes may, in part, reflect variations in the apparent and state lifetimes (Supplementary Fig. 2c).

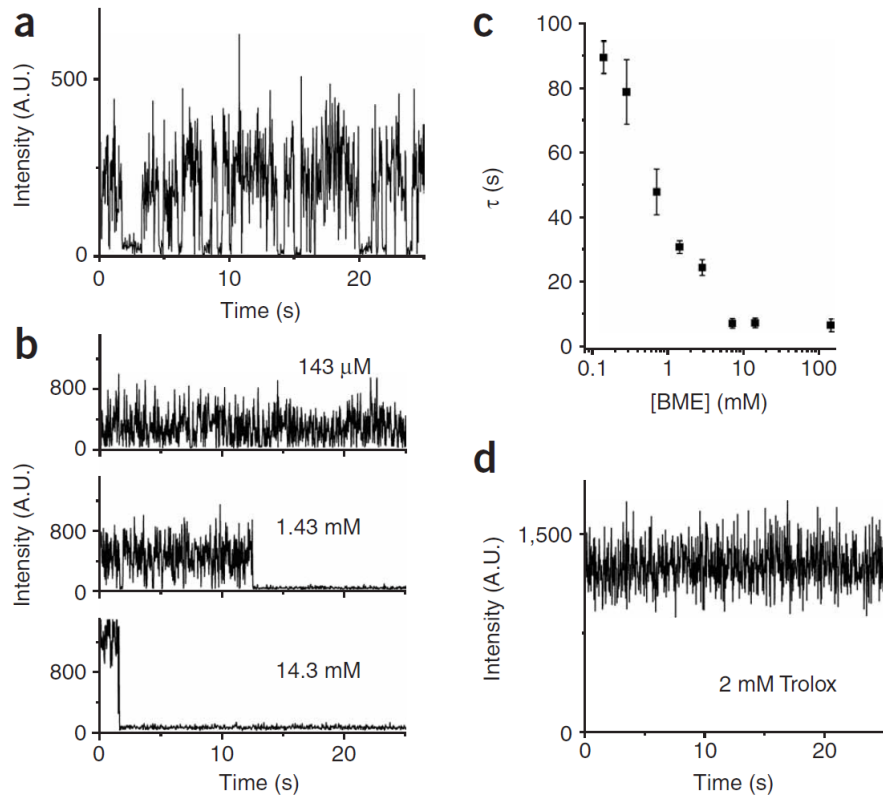
To evaluate these fluorophore derivatives, we tracked each compound with an atomic force microscope (AFM) in a buffer solution, dye-doped DNA oligonucleotides that we perfused by homeostasis using hydrophobic interaction chromatography (Online Methods). Using wide-field, total internal reflection fluorescence (TIRF) imaging<sup>11</sup>, we assessed the photophysical properties of our fluorophore-linked fluorophores (COT, NBA) at the single-molecule scale under direct laser illumination at 640 nm. We used typical single-molecule imaging conditions with very low ionic strength scavenging system to remove molecular cations from solution<sup>12</sup>.

<sup>1</sup>Department of Chemistry and Biochemistry, Weill Medical College of Cornell University, New York, New York, USA. <sup>2</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>3</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>4</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>5</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>6</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>7</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>8</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>9</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>10</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>11</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA. <sup>12</sup>Department of Chemistry, Cornell University, Ithaca, New York, USA.

RECEIVED 10 MAY 2011 | ACCEPTED 10 SEPTEMBER 2011 | PUBLISHED ONLINE 1 JULY 2012 | DOI:10.1038/NMETH1274

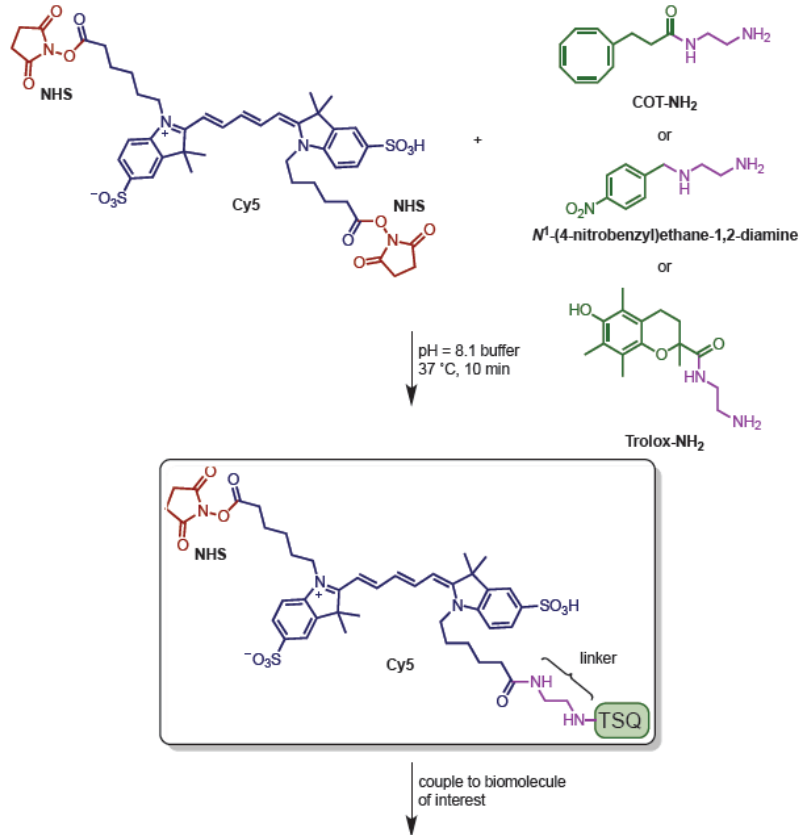
68 | VOL.9 NO.1 | JANUARY 2012 | NATURE METHODS

# Photoblinking and photobleaching



Rasnik et al., 2006

# Cyanine fluorophore derivatives



# Cyanine fluorophore derivatives

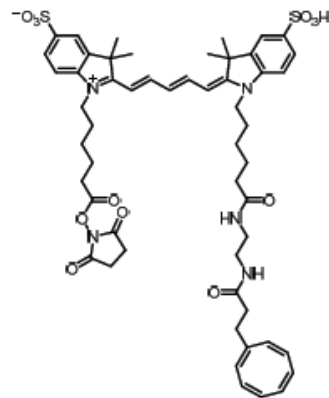
## COT-Cy5-NHS:

LCMS: 25-65% B over 2.5 min, rt = 1.73 min

HPLC: 25-65% B over 25 min, rt = 13.01 min

ESI-MS:  $m/z$  calculated for  $C_{54}H_{65}N_5O_{12}S_2$

$[M+H]^+$  1040.4, found 1040.7



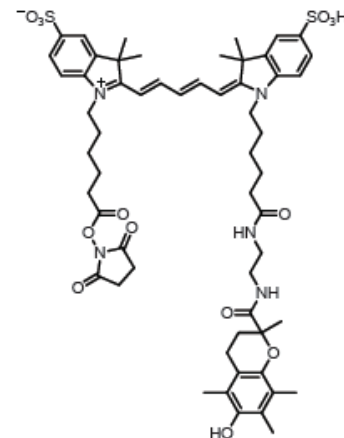
## TX-Cy5-NHS:

LCMS: 25-65% B over 2.5 min, rt = 1.80 min

HPLC: 25-65% B over 25 min, rt = 13.65 min

ESI-MS:  $m/z$  calculated for  $C_{57}H_{71}N_5O_{14}S_2$

$[M+H]^+$  1114.5, found: 1114.8



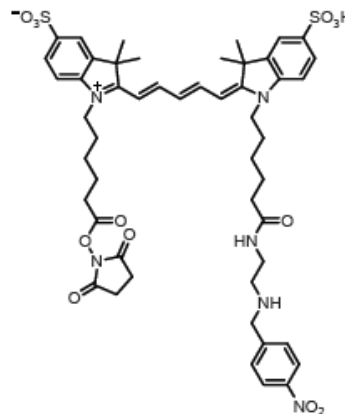
## NBA-Cy5-NHS:

LCMS: 25-65% B over 2.5 min, rt = 1.28 min

HPLC: 25-65% B over 25 min, rt = 7.33 min

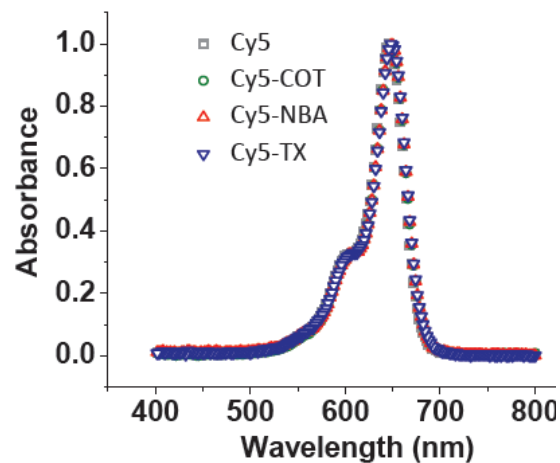
ESI-MS:  $m/z$  calculated for  $C_{50}H_{60}N_6O_{13}S_2$

$[M+H]^+$  1017.4, found: 1017.7

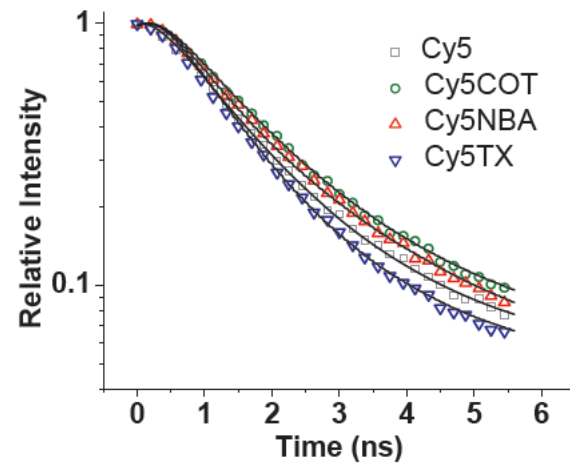
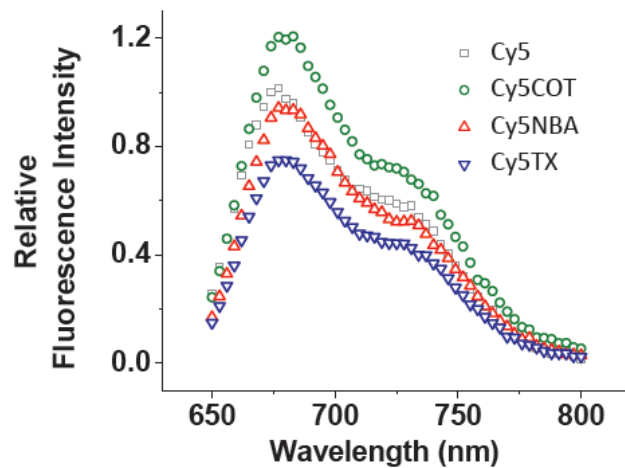


# Cyanine fluorophore derivatives

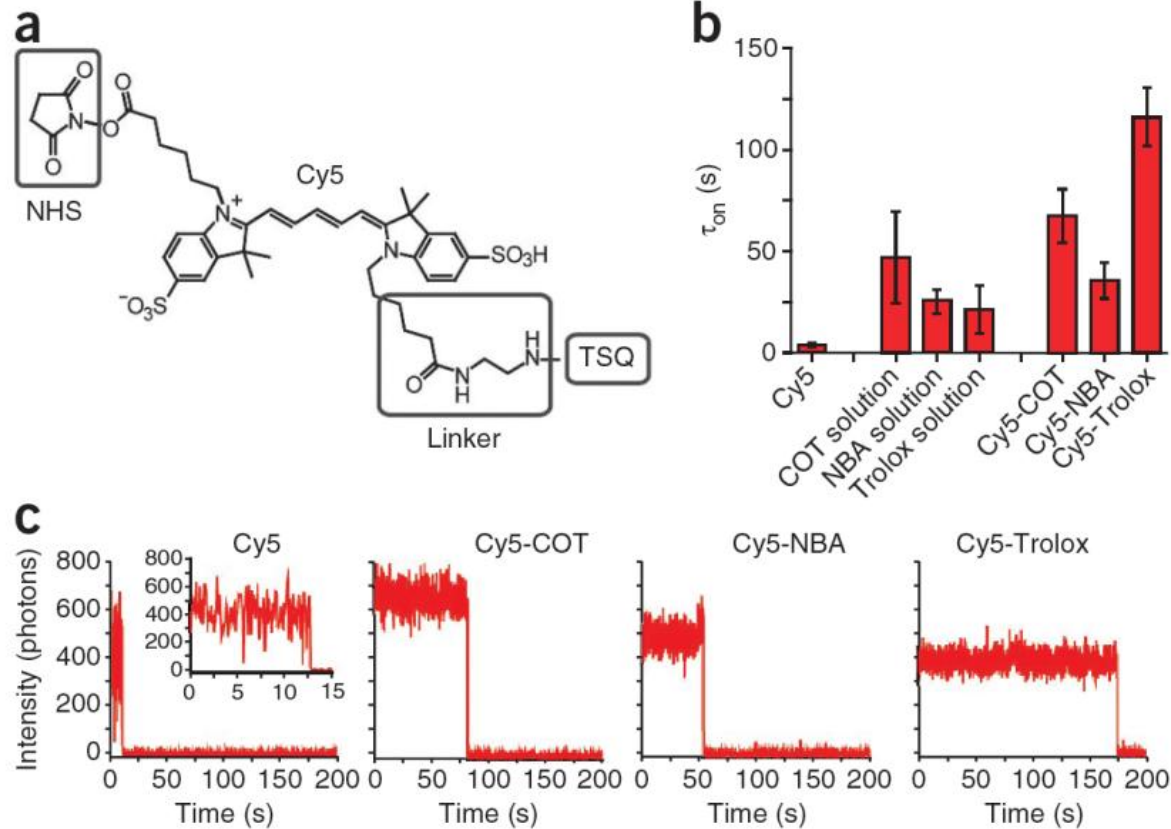
a



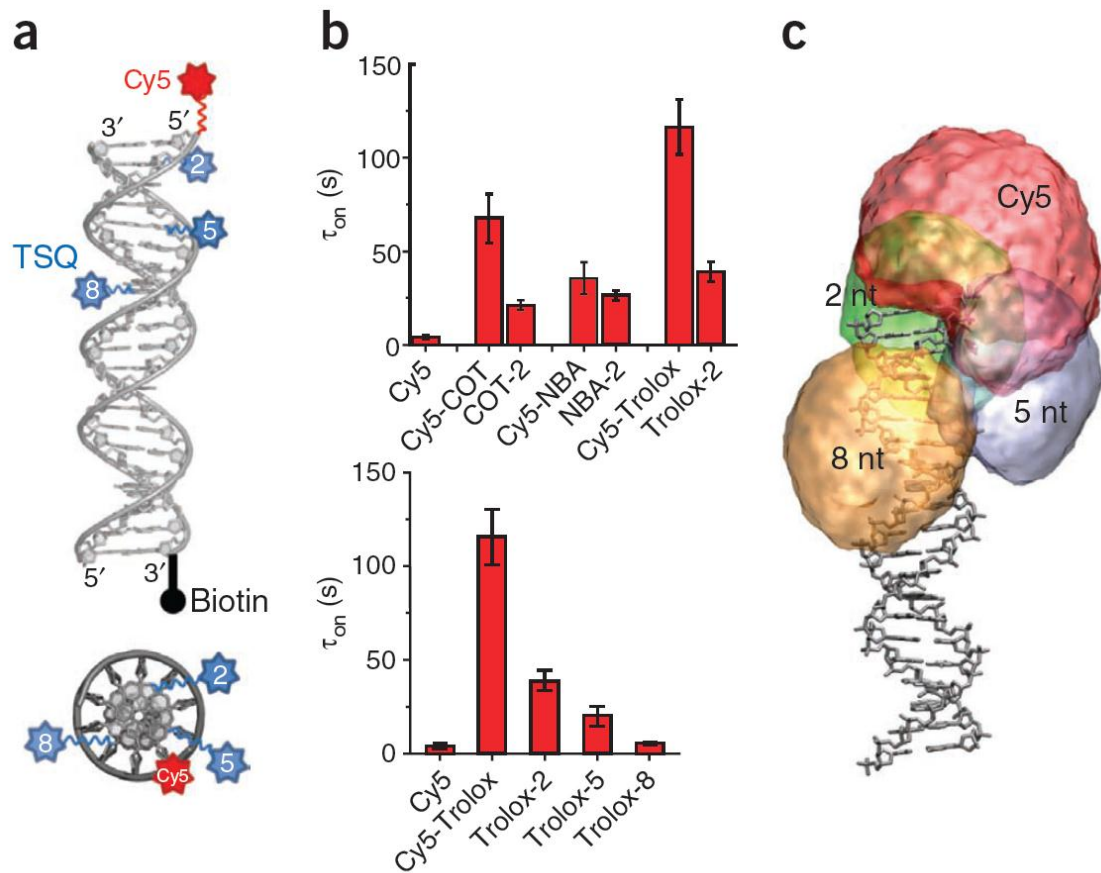
b



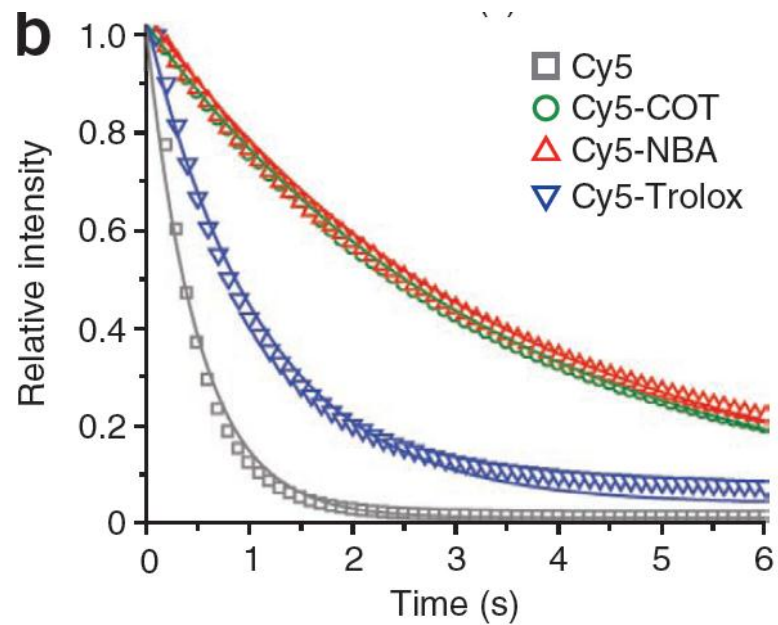
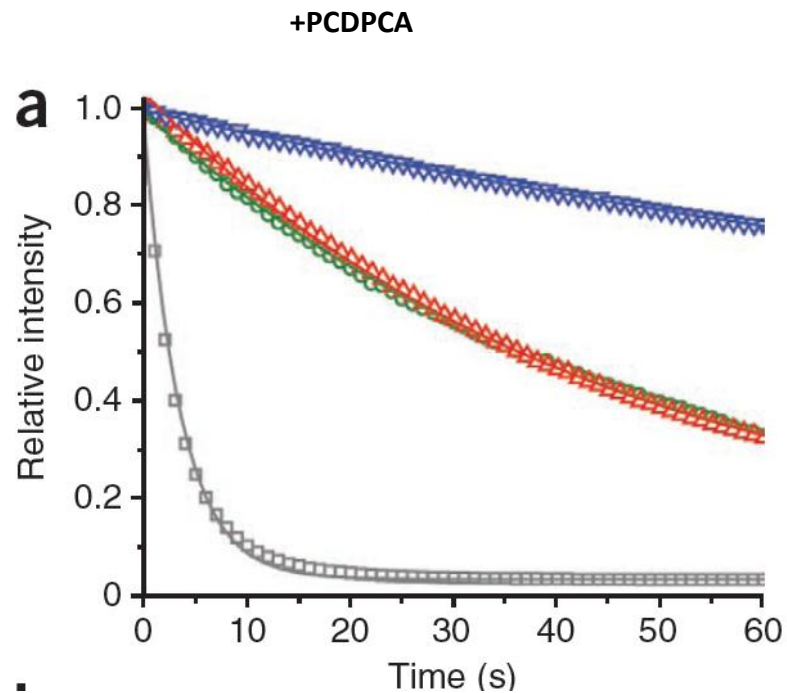
# Single molecule traces



## distance control



## With Oxygen scavenging system





on the living cell

