

A EUROPEAN JOURNAL OF CHEMICAL BIOLOGY

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SYNTHETIC BIOLOGY & BIO-NANOTECHNOLOGY

Accepted Article

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To be cited as: *ChemBioChem* 10.1002/cbic.201700286

Link to VoR: <http://dx.doi.org/10.1002/cbic.201700286>

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Modulation of Mitochondriotropic Properties of Cyanine Dyes by *In Organello* Copper-Free Click Reaction

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Abstract: Cyanine dyes show a general propensity to localize to polarized mitochondria. This mitochondriotropism was used to perform a copper-free click reaction in the mitochondria of living cells. The *in organello* reaction of a Cy3 and Cy5 dye led to a product that was easily traceable by Förster resonance energy transfer (FRET). As determined by confocal laser scanning microscopy, the Cy3-Cy5 conjugate showed enhanced retention in mitochondria as compared to the starting compounds. This enhancement of a favorable property can be achieved by synthesis *in organello* but not outside mitochondria.

Keywords: click chemistry • fluorescent probes • FRET • *in organello* synthesis • mitochondrial targeting

Targeting of small molecules to active mitochondria is driven by a fundamental desire to understand the workings of the eukaryotic cell's powerhouse, e.g. by delivery of chemical probes for reactive species associated to mitochondrial metabolism. To achieve this goal, basic research focuses on the requirements of a small molecule to acquire mitochondriotropic characteristics,^[1] i.e. the property of localizing to mitochondria. To date, a broad range of molecules, including many fluorescent dyes, have been identified as mitochondriotropic.^[2] Common dominators for mitochondriotropism include a delocalized positive net charge, an overall lipophilic character, and a certain proportion of aromatic character in a small molecule's scaffold.^[3] For instance, derivatives of triphenylphosphonium (TPP) cations^[3-4] are widely used to achieve mitochondrial localization. The accumulation in polarized mitochondria is driven by the mitochondrial membrane potential ($\Delta\Psi_m$) - the gradient of charges and protons across their inner membrane. Several therapeutic concepts and detection approaches rely on $\Delta\Psi_m$ to pull small molecules across the inner membrane and enrich them for *in organello* reactions. On one hand, numerous

publications report on probes for a specific reaction with an endogenous mitochondrial component, aiming at either modulation of the mitochondrial genome or protein pool or measurement of reactive oxygen species (ROS) and metals.^[2] On the other hand, mitochondrial import of two exogenous reaction partners has recently been shown.^[5] Logan *et al.* performed a strain-promoted azide-alkyne cycloaddition^[6] (SPAAC) reaction of two mitochondriotropic molecules in mitochondria.^[5a] The rate of product formation responded to the plasma and mitochondrial membrane potential dependent accumulation of its building blocks and was therefore used to assess changes in $\Delta\Psi_m$. Also, Tomas-Gamasa *et al.* reported importing a ruthenium complex to mitochondria, which exposed its catalytic activity by uncaging a second, mitochondria-targeted probe - in this case a rhodamine dye.^[5c]

A desirable outcome for the *in organello* formation of a new chemical entity is synergy, or some other flavor of added value. Unless scientists can show that there is an advantage to separately targeting reactants over administering the product directly, the field is unlikely to move beyond basic fundamental research and affect science in the broader sense. We focused on intracellular distribution, i.e. the degree of mitochondriotropism, as a likely parameter to yield such synergy. In this regard, we ventured to develop cyanine structures^[7] as targeting moieties to direct the transport of reactive structures, such as strained alkynes and azide groups, to the mitochondria. Cyanine dyes by themselves are promising candidates because the positive charge favors import across the inner mitochondrial membrane driven by $\Delta\Psi_m$. We observed high colocalization with a *bona fide* Mitotracker for a series of lipophilic cyanine dyes with Cy3 or Cy5 scaffolds^[8] (Pearson's Coefficient > 0.9; see Figure S7 and Table S1), as long as these were not sulfonated (not shown). We conclude that lipophilic cyanine dyes are, as a scaffold, inherently mitochondriotropic and set out to exploit this feature for a reaction *in organello*.

While our studies were underway, the mitochondriotropism of cyanine dyes was exploited to measure levels of ROS in living cells.^[9] Jia and coworkers reported the loss of FRET from a Cy3-Cy5 construct upon specific oxidation of Cy5 by peroxynitrite. Based on this reported FRET effect of a covalent Cy3-Cy5 conjugate observed *in organello*,^[9] we decided to use this effect as a readout for successfully SPAAC mediated coupling of two dyes in mitochondria. We therefore synthesized a dibenzocyclooctyne (DBCO) functionalized Cy3 dye **3** by conjugating a Cy3 chromophore that carries a carboxylic acid group **1** with commercially available DBCO-amine **2** (Figure 1A). A reaction of Cy3-DBCO **3** and Cy5-azide **4** was analyzed by HPLC-ESI-MS. It led to the identification of the dicationic SPAAC product **5** (Figure 1B) by its m/z of 647.4 ([M]⁺⁺, calc.: 647.39, see Figure S8). The excitation and emission spectra of Cy3-DBCO **3** and Cy5-azide **4** are shown in Figure 1C. The

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spectrum of SPAAC product **5** (Figure 1D) exhibits near-complete quenching of donor emission and high acceptor emission with a maximum at 667 nm when excited at Cy3 donor excitation wavelength. These spectral characteristics are attributed to FRET. The reaction kinetics was analyzed by monitoring the decrease of donor emission caused by FRET due to the formation of the product **5**. At a 10 μM concentration of **3** and **4** (in PBS, 40% DMSO at 37°C), a 50% decrease of donor emission followed by saturation was observed within the first one hour (Figure S9).

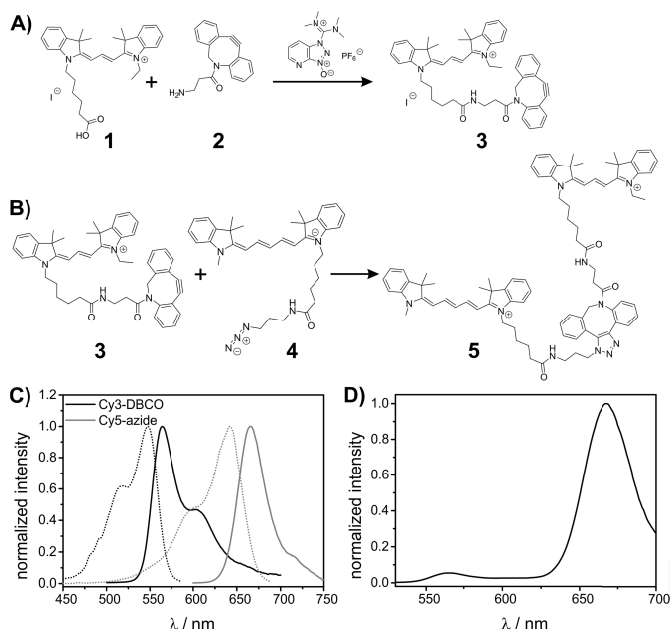


Figure 1. A) Synthetic route for the preparation of Cy3-DBCO **3**. B) Reaction between Cy3-DBCO **3** and Cy5-azide **4** leading to the formation of SPAAC product **5**. C) Excitation (dotted line) and emission (solid line) spectra of Cy3-DBCO **3** and Cy5-azide **4** in PBS, 40% DMSO. D) Emission spectrum of product **5** at 488 nm excitation.

To evaluate mitochondrial accumulation of Cy3-DBCO **3** and Cy5-azide **4** in cell culture, rat brain endothelial (RBE4) cells were treated with each dye separately and imaged on a Zeiss LSM 880 confocal microscope. In a series from 12.5 nM to 1 μM a concentration of 250 nM was found optimal as it revealed high mitochondrial specificity and good signal detection. For concentrations higher than 250 nM a diffusive cytosolic fluorescence was observed in addition to the mitochondrial staining (not shown). Furthermore, at 250 nM of **3** and **4** SPAAC product formation over 12 hours was negligible for the in-cuvette reaction (Figure S9). Thus, any detectable mitochondrial SPAAC product in the same time frame would be indicative of an increased reaction rate as a consequence of locally increased concentrations. Mitochondrial fluorescence signal reached steady state levels after about 2h of incubation. Importantly, at an excitation wavelength of 488 nm neither cross excitation nor bleed through were detected in the FRET channel (Figure S10). A heterogeneous distribution of fluorescence intensity of individual cells was attributed to varying $\Delta\Psi_m$ between cells.^[10] The dependence of mitochondrial accumulation on $\Delta\Psi_m$ was confirmed by loss of fluorescence signal after co-incubating the cells with 5 μM of the respiratory uncoupler carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) (Figure S11C+D).^[11] When removing extracellular dye by washing the cells with PBS

and subsequent incubation in fresh dye-free cell medium, mitochondrial fluorescence decreased. This decrease indicates that the monocationic dyes re-equilibrate across the plasma and mitochondrial membrane. After a second washout step, the fluorescence signal was reduced to undetectable levels (Figure S11E+F). We conclude that (i) staining of polarized mitochondria by cyanine dyes is reversible, that (ii) the dynamics of mitochondrial enrichment as well as of diffusion from mitochondria fall within the time frame of this protocol, and that (iii) the latter is therefore suitable to assess the dynamic intracellular distribution of small cyanine dyes.

To investigate if the compounds would react inside the mitochondria RBE4 cells were incubated for four hours with **3** and **4** (Figure 2A). As a control, a mock incubation with azides of both dyes was conducted (Figure 2B). In a second control reaction, SPAAC reagents were used, but $\Delta\Psi_m$ was diminished by FCCP (Figure 2C). Finally, an incubation with the preformed product **5** was conducted in parallel (Figure 2D). Of note, because a FRET signal observed in the mock incubation (Figure 2B) disappeared after application of the washout protocol, it presumably arose from enriched concentrations of unreacted dyes. After washout, only the SPAAC reaction revealed an appreciable FRET signal, which we therefore conclude to have originated in the organelle. A semi-quantitative image analysis by manual cell segmentation showed a significantly higher signal for the *in organello* reaction than for all control and parallel experiments (Figure 2E). We conclude that FRET signal in mitochondria under these conditions requires (i) membrane potential, (ii) application of two SPAAC-reaction partner molecules with cyanine moieties for organellar targeting and (iii) that the preformed product **5** is slower to localize to mitochondria than either of the reaction partners.

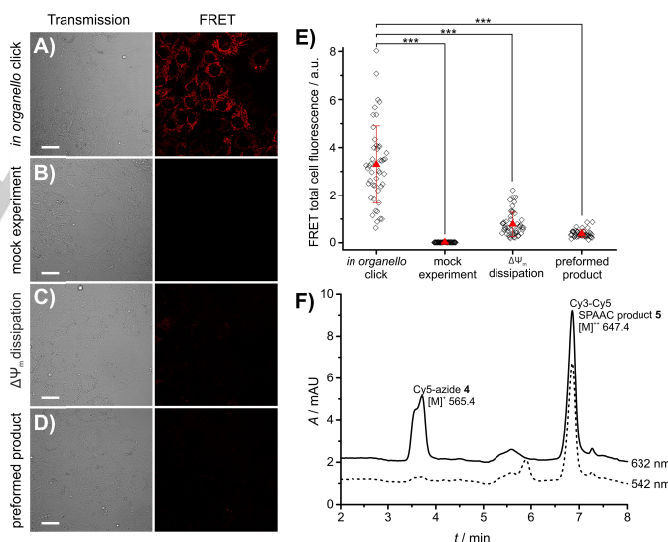


Figure 2. Confocal images for synchronous Cy3 and Cy5 incubation (250 nM each). Cells were treated for four hours followed by two washout cycles. FRET channel: Cy5 signal upon excitation at 488 nm. Scale bar: 25 μm . A) *In organello* SPAAC with Cy3-DBCO **3** and Cy5-azide **4**. B) Mock experiment with Cy3-azide and Cy5-azide **4**. C) *In organello* SPAAC in the presence of 5 μM FCCP. D) Preformed product **5**. E) FRET total cell fluorescence for manually segmented cells treated with conditions A)-D) ($N \sim 50$ cells, mean \pm SD). Student's two sample t-test: *** $p \leq 0.001$. F) Compounds were extracted from cells after *in organello* SPAAC followed by two washout cycles and separated on an RP-HPLC. Coupling with MS detection allowed assignment of peaks to Cy5-azide **4** and product **5**. Peak (5.9 min) in the 542 nm absorbance trace is an unknown side product of Cy3.

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To further demonstrate the formation of product **5**, we adapted^[5a, 12] an extraction protocol for the recovery of fluorescent molecules from tissue culture. LC-MS analysis of the re-isolated fluorescent compounds (Figure 2F) identified the product **5**, confirming the interpretation that the observed FRET signal was due to an *in organello* reaction.

We hypothesized that it should be possible to perform an *in organello* reaction also with reversed functionalities as represented by Cy3-azide **6** and Cy5-DBCO **7** (Figure 3A). An LC-MS analysis of the reaction mixture of **6** and **7** in solution identified the corresponding SPAAC product **8** at m/z 661.8 ($[M]^{++}$, calc: 661.41, see Figure S12A). In-cuvette characterization of **6** and **7** showed excitation and emission characteristics similar to their counterparts **3**, **4** (Figure S12B). Furthermore, reaction kinetics at 10 μ M and 250 nM concentration were monitored by fluorescence (Figure S13). Surprisingly, the reaction velocity of **6** and **7** at 10 μ M concentration was reduced in comparison to that of Cy3-DBCO **3** and Cy5-azide **4** (Figure 3B), as can easily be followed by the slower decrease in donor emission of **6**. Here, a 50% reduction in donor emission was observed within four hours in contrast to one hour for product **5** formation. Consistent with the other cyanine dyes, we observed mitochondrial staining (Figure S14A+B) for Cy3-azide **6** and Cy5-DBCO **7** that was reduced when cells were co-treated with FCCP (Figure S14C+D) or subjected to the washout procedure (Figure S14E+F). We furthermore investigated if simultaneous administration of **6** and **7** to the cell medium would lead to product **8** formation in the mitochondria of RBE4 cells. The detection of FRET signal in the SPAAC reaction conditions (Figure S15) but not in the control experiments confirmed that the *in organello* reaction proceeded with these "reversed" SPAAC functionalities.

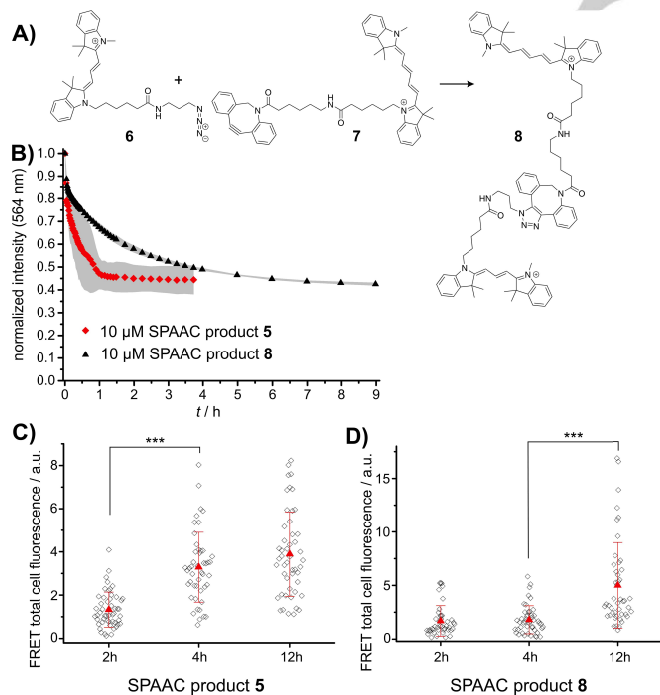


Figure 3. A) Reaction between Cy3-azide **6** and Cy5-DBCO **7** forming SPAAC product **8**. B) In cuvette kinetics of SPAAC product **5** and **8** formation. Reactants were mixed at 10 μ M concentration. Donor emission at 564 nm was normalized to intensity at starting concentration and plotted as mean \pm SD (in gray) of three independent experiments. C), D) FRET total cell fluorescence for manually segmented cells ($N \sim 50$ cells, mean \pm SD). RBE4 cells were treated with 250 nM concentrations of SPAAC reaction partners (C) Cy3-DBCO **3** and

Cy5-azide **4**, leading to SPAAC product **5**; D) Cy3-azide **6** and Cy5-DBCO **7**, leading to SPAAC product **8** for two, four or twelve hours followed by two washout cycles. Student's two sample t-test: *** $p \leq 0.001$.

Remarkably however, the SPAAC reaction leading to **5** proceeds significantly faster than that with inverted functionalities leading to **8**. As is clearly apparent in the comparative kinetics in Figure 3B, the dynamic range spans only one hour in the former, but four hours in the latter case. To verify if this effect also took place in cell culture, we determined in which intervals of incubation with the starting compounds the strongest dynamics (*i.e.* an increase) of the FRET signal occurred. As shown in Figure 3C, the strongest increase of *in organello* formation of **5** took place in the first 4 hours of incubation (see also Figure S16A). In contrast, the FRET signal from **8** (Figure 3D) kept increasing during the first 12 hours (Figure S16B). Thus, we found that certain characteristics of the SPAAC reaction kinetics observed in the cuvette translate to the observation of the *in organello* reaction.

In conclusion, we state that this is the first report on the covalent formation of a FRET dye inside mitochondria. Importantly, this study points out the advantages of an *in organello* SPAAC reaction conducted with FRET pairing reaction partners. The Cy3-Cy5 SPAAC products possess synergistic properties that are not present in their building blocks, namely a characteristic FRET effect and an altered membrane permeability. The latter is due to the molecular characteristic of two delocalized cationic charges potentially leading to lower membrane permeation and greater retention in the negative mitochondrial matrix than observed for the monocationic SPAAC-precursors.^[13] The efficient uptake of the starting compounds in combination with increased mitochondrial retention of the SPAAC products thus constitutes an advantage of conducting the reaction *in organello*, rather than applying the preformed SPAAC product to living mitochondria. A strong accumulation of the Cy3-Cy5 SPAAC product in mitochondria as shown here is made possible by $\Delta\Psi_m$ -dependent import of its lipophilic precursors and subsequent *in organello* reaction. Thus, our studies generated a washout-resistant FRET dye inside mitochondria which will be interesting to apply *e.g.* to ROS imaging^[9] with reduced probe concentration and increased mitochondrial specificity or improved visualization of mitochondria with high respiration competency across a diverse population.

Acknowledgements

This work was financially supported by the MPG with the Johannes Gutenberg University Mainz (I.N.) and the DFG SFB 1066 (projects A7, Q2). We acknowledge the NMR platform at the University Jena for support in NMR spectroscopy.

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