Clickable fluorescent dyes for multimodal bioorthogonal imaging†‡

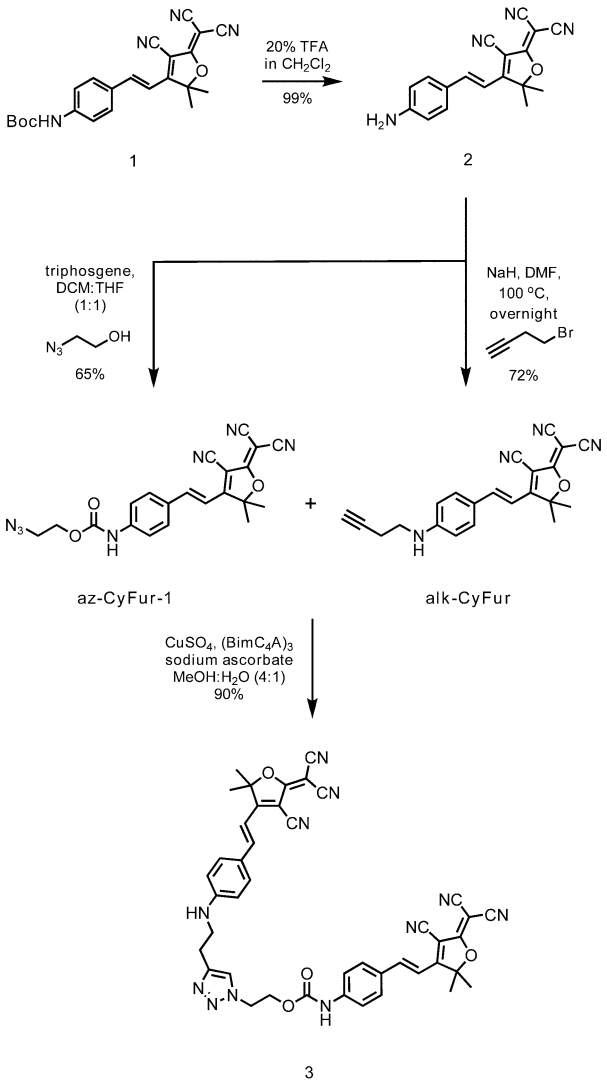
Lun K. Tsou, Mingzi M. Zhang and Howard C. Hang*

Received 18th August 2009, Accepted 18th September 2009
First published as an Advance Article on the web 26th October 2009
DOI: 10.1039/b917119n

Bioorthogonal ligation with functionalized fluorescent dyes enables visualization of nuclei acids, proteins and metabolites in biological systems. Bright and modular azide- and alkyne-functionalized dyes are therefore needed to expand the fluorescence imaging capabilities of bioorthogonal ligation methods. We describe the concise synthesis of clickable fluorescent dyes based on 2-dicyanomethylene-3-cyano-2,5-dihydrofuran fluorophores and demonstrate their utility for multicolor imaging of azide- and alkyne-modified proteins as well as FRET studies.

Bioorthogonal ligation of fluorescent dyes and affinity tags onto biomolecules has afforded new opportunities to investigate cellular pathways with chemical tools.[1,2] Notably, the development of chemical reporters functionalized with azides or alkynes has enabled the fluorescence imaging of nucleic acid and protein synthesis as well as posttranslational modifications (PTMs) using the Staudinger ligation or click chemistry.[1] Moreover, chemical reporters enable dynamic imaging of biomolecules in cells and *in vivo* by pulse-labeling in combination with functionalized-fluorophores with orthogonal spectral properties.[3–6] The ability to separate the biochemical reactivity of mechanism-based probes from imaging/affinity reagents also allows the detection of specific enzymatic activities in living cells.[7,8] Development of modular fluorescent dyes compatible with bioorthogonal ligation methods will expand the repertoire of reagents for diverse imaging applications using mechanism-based probes or chemical reporters. Herein, we report a concise synthesis of clickable fluorescent dyes based on 2-dicyanomethylene-3-cyano-2,5-dihydrofuran (DCDHF) fluorophores for multimodal imaging applications using Cu(I)-catalyzed azide-alkyne cycloaddition (CuAAC).[2]

For fluorescent detection of alkyne- and azide-labeled proteins, we synthesized a new set of clickable fluorescent dyes based on DCDHF fluorophores, given their photostability for single-molecule imaging and tunable red-shifted fluorescent emission properties.[9–11] Condensation of *tert*-butyl 4-formylphenylcarbamate[12] and 3-cyano-2-dicyanomethylene-4,5,5-trimethyl-2,5-dihydrofuran[13] afforded compound **1** in 72% yield (Scheme 1). Removal of *t*-Boc group and alkylation of compound **2** with 1-bromobutyne gave alkynyl-DCDHF derivative (alk-CyFur) in 72% yield (Scheme 1). Acylation of DCDHF fluorophore **2** with alkyl-azide substrates afforded az-CyFur-1 and az-CyFur-2 in 65% and 57% yield, respectively (Scheme 1 and Supporting Information Fig. S1). While alk-CyFur exhibited absorption (abs)/emission (em) maxima at 580 nm/640 nm, acylated-DCDHF derivatives (az-CyFur-1 and az-CyFur-2) yielded abs/em maxima at 470 nm/580 nm (Fig. 1A and Supporting Information Fig. S1B and S1C). The differential fluorescence properties of N-acylated compared to N-alkylated DCDHF derivatives are consistent with previous studies demonstrating that capping of the aniline functionality quenches the red-shifted emission of DCDHF fluorophore **2**.[9–11] The quantum yields of all three clickable CyFur dyes are similar to previously described



Scheme 1 Synthesis of clickable CyFur dyes.

The Laboratory of Chemical Biology and Microbial Pathogenesis, The Rockefeller University, New York, NY 10065, USA. E-mail: hhang@mail.rockefeller.edu

† This paper is part of an *Organic & Biomolecular Chemistry* web theme issue on protein labelling and chemical proteomics. Guest editors: Ed Tate and Matthew Bogyo.

‡ Electronic supplementary information (ESI) available: Synthetic methods, supplementary figures (Fig. S1–Fig. S4) and spectra. See DOI: 10.1039/b917119n

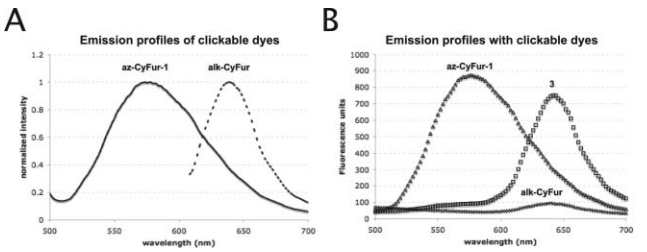


Fig. 1 Spectral properties of clickable CyFur dyes. (A) Emission spectra of clickable CyFur dyes. Emission spectra were acquired at excitation of 470 nm and 580 nm for az-CyFur-1 (solid line) and alk-CyFur (dash line), respectively. (B) Emission spectra of alk-CyFur (×), az-CyFur-1 (Δ) and fluorescent adduct 3 (□), excitation at 410 nm.

Table 1 Optical properties of clickable CyFur dyes

	λ_{max} [a] (nm)	λ_{max} [a] (nm)	ε_{max} [b] ($M^{-1}cm^{-1}$)	Φ_F [c]
alk-CyFur	580	640	33 933	0.0147
az-CyFur-1	470	580	20 533	0.0067
az-CyFur-2	470	580	12 100	0.0027

[a] Spectra were obtained in DMSO. [b] Measurements were done in MeOH. Extinction coefficients at 470 nm for az-CyFur-1, az-CyFur-2 and at 580 nm for alk-CyFur are averaged over three independent experiments. [c] Quantum yields referenced against cresyl violet ($\Phi_F = 0.54$ in MeOH). Alk-CyFur was excited at 580 nm. Both az-CyFur-1 and az-CyFur-2 were excited at 470 nm. Experimental details are described in Supporting Information.

N-alkylated and quenched DCDHF derivatives (Table 1).[9–11] In accordance with other reported DCDHF-based fluorophores,[9–11] the fluorescence emission of CyFur dyes increases significantly in a more viscous or rigid environment such as glycerol (Supporting Information Fig. S2). Based on the observed absorption and emission spectra of az-CyFur-1 and alk-CyFur, dimerization of these two fluorophores was predicted to undergo Förster resonance energy transfer (FRET). Indeed, coupling of az-CyFur-1 and alk-CyFur *via* CuAAC afforded clicked fluorophore **3**, which exhibited FRET between the acylated- and alkylated-DCDHF derivatives (Fig. 1B). Excitation of fluorophore **3** at 410 nm resulted in strong fluorescence emission at 640 nm, whereas an unreacted 1:1 mixture of az-CyFur-1:alk-CyFur afforded similar spectral properties to the dyes alone (Fig. 1B and Supporting Information Fig. S3). These results demonstrate that differential modification of DCDHR fluorophores provides clickable red-shifted fluorescent dyes with tunable spectral properties that can also function as donor and acceptor FRET pairs.

To explore the utility of these clickable CyFur dyes for imaging azide- and alkyne-modified proteins *in vitro* and in cells, we employed azido- and alkynyl-fatty acids chemical reporters to metabolically label *N*-myristoylated and *S*-palmitoylated proteins.[14,15] Jurkat T cell lysates labeled with azido-fatty acids (az-12, az-15) were subsequently reacted with alk-CyFur or alk-Rho *via* the CuAAC and separated by gel-electrophoresis (Fig. 2). Labeled proteins were visualized by in-gel fluorescence scanning at various excitation/emission channels to detect CyFur- and Rho-modified proteins. Fluorescence imaging at 633 nm excitation and 670 nm emission allowed selective detection of alk-CyFur-labeled proteins analogous to the profile of fatty-acylated proteins visualized with alk-Rho (excitation 532 nm/emission 580 nm) (Fig. 2).[14] Similarly, Az-CyFur-1 allows selective fluorescent imaging of alk-12-labeled cell lysates (Supporting Information Fig. S4). The near-infrared fluorescent properties of alk-CyFur enabled profiling of azide-modified proteins in gels with minimal spectral overlap to acylated-CyFur and Rho fluorophores (Fig. 2 and Supporting Information Fig. S4).

The clickable and environmentally-sensitive CyFur dyes also allow robust fluorescent imaging of azide- and alkyne-labeled proteins in cells. HeLa cells were metabolically labeled with az-12 or alk-12, fixed/permeabilized, reacted with alk-CyFur, az-CyFur-1 or az-Rho and imaged as previously described.[14] Az-12 and alk-12-labeled HeLa cells yielded significantly higher levels of fluorescent labeling with alk-CyFur and az-CyFur-1, respectively, compared to DMSO control using settings for fluorescein dyes (excitation 488 nm/emission 560 nm) (Fig. 3A). In contrast, image settings for red-emitting Cy5 dyes (excitation 630 nm/emission 650 nm) enables visualization of alk-CyFur-labeled cells with no crosstalk to az-CyFur-labeled cells (Fig. 3B). CyFur-labeled proteins were concentrated within intracellular membranes and excluded from the nuclei, which is in accordance with previous imaging studies of fatty-acylated proteins[14,16] and

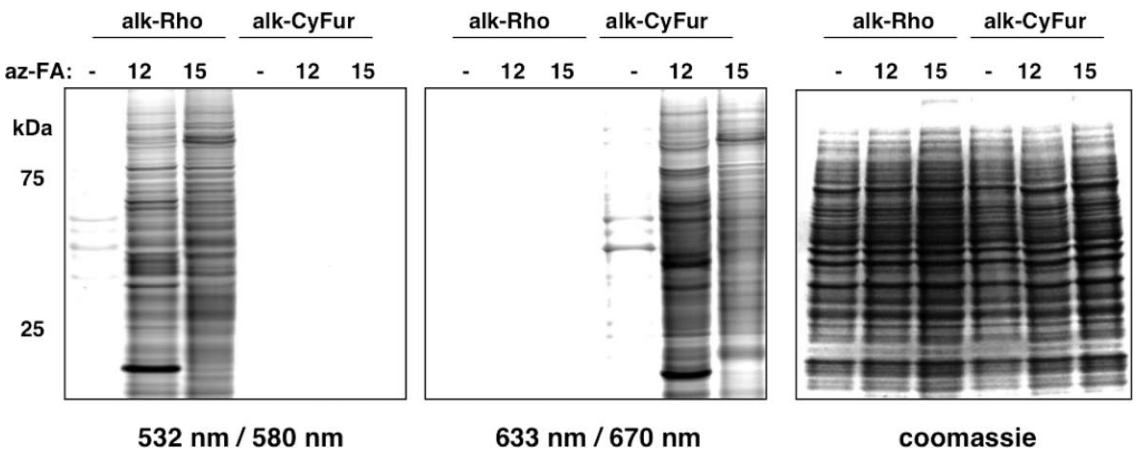


Fig. 2 In-gel fluorescence imaging of azido-fatty acid (az-FA)-modified proteins from metabolically labeled Jurkat T cells after bioorthogonal ligation with clickable CyFur dyes.

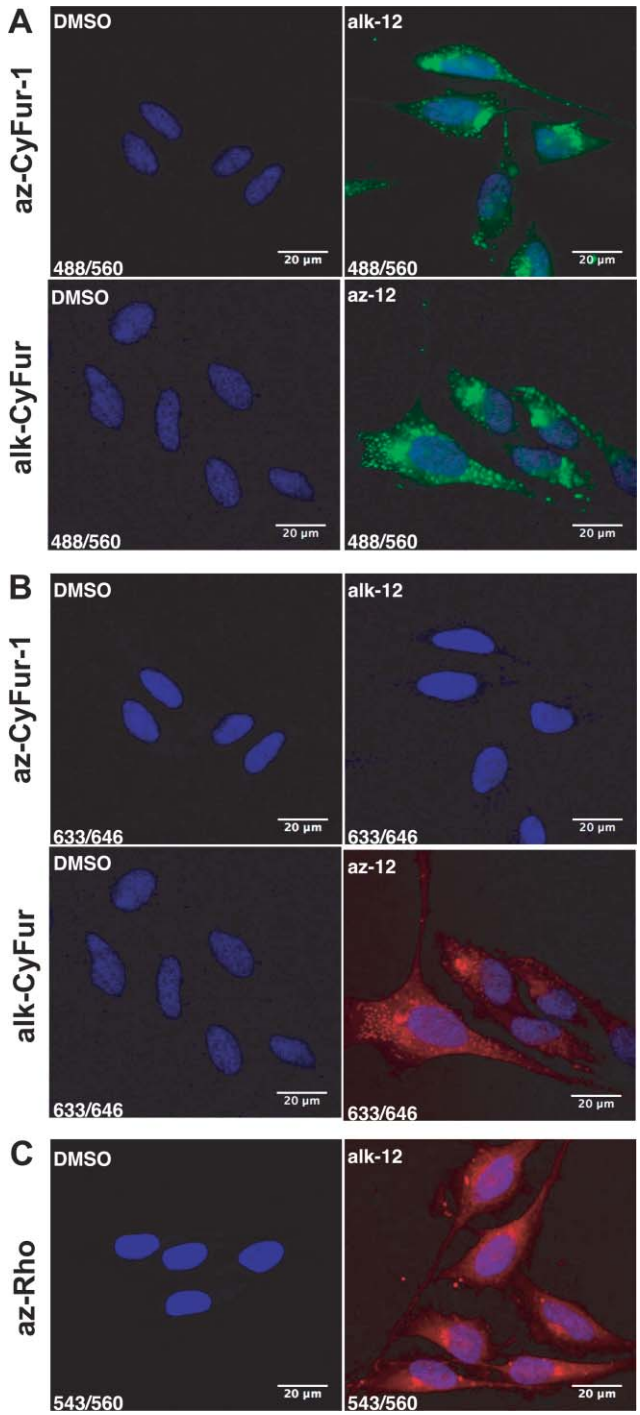


Fig. 3 Fluorescence microscopy of azido (az-12)- and alkynyl (alk-12)-fatty acid labeled HeLa cells after bioorthogonal ligation with clickable dyes. (A) Imaging with 488 nm excitation and 560 nm emission long-pass filter. Top panel: az-CyFur-1-labeled proteins. Bottom panel: alk-CyFur-labeled proteins. (B) Imaging with 633 nm excitation and 646-753 nm emission filter. Top panel: az-CyFur-1-labeled proteins. Bottom panel: alk-CyFur-labeled proteins. (C) Imaging of az-Rho-labeled proteins. 543 nm excitation and 560-615 nm emission filter. DAPI imaging in all panels was performed by 405 nm excitation and 420-480 nm emission filter.

similar to that observed for cellular labeling with az-Rho done in parallel (Fig. 3C). Interestingly, fatty acid chemical reporter labeled cells visualized with clickable CyFur dyes yielded more punctate intracellular membrane structures compared to Rho dyes, which may be due to solvatochromism for hydrophobic environments reported for DCDHF fluorophores.[9,11]

In summary, we have synthesized a new set of clickable fluorescent dyes based on the DCDHF fluorophores for bioorthogonal ligation applications. Synthesis of these clickable CyFur dyes is efficient, modular and scalable, which enables facile access to azide- or alkyne-modified fluorophores with different spectral properties by alkylation or acylation of single common DCDHF fluorescent precursor. The in-gel fluorescence scanning and cellular imaging studies of azide- and alkyne-modified fatty-acylated proteins showcase the utility of the clickable CyFur dyes for imaging endogenously expressed proteins. The fluorescent property of alk-CyFur complements previously reported clickable near-infrared dyes[17–19] and should facilitate dual imaging of chemical reporters as well as *in vivo* imaging applications in the future. Additionally, the spectral overlap of the acylated- and alkylated-CyFur dyes yields useful donor and acceptor pairs for further FRET studies. The clickable CyFur dyes reported here provide alternative and readily accessible reagents for multimodal fluorescence imaging applications using bioorthogonal chemical probes/reporters to study cellular pathways.

Acknowledgements

The authors thank Dr. Anuradha Raghavan, Guillaume Charron and Paul Dossa for providing fatty acid chemical reporters and clickable Rho dyes. L. K. T. acknowledges support from The Rockefeller University Marie-Josée and Henry Kravis Postdoctoral Fellowship. M. M. Z. is supported by A*STAR, Singapore. H. C. H. acknowledges support from The Rockefeller University, Irma T. Hirschl/Monique Weill-Caulier Trust and Ellison Medical Foundation.

Notes and References

1 E. M. Sletten and C. R. Bertozzi, *Angew. Chem., Int. Ed.*, 2009, **48**, 6974.
2 M. Meldal and C. W. Tornoe, *Chem. Rev.*, 2008, **108**, 2952.
3 J. M. Baskin, J. A. Prescher, S. T. Laughlin, N. J. Agard, P. V. Chang, I. A. Miller, A. Lo, J. A. Codelli and C. R. Bertozzi, *Proc. Natl. Acad. Sci. U. S. A.*, 2007, **104**, 16793.
4 S. T. Laughlin, J. M. Baskin, S. L. Amacher and C. R. Bertozzi, *Science*, 2008, **320**, 664.
5 P. V. Chang, J. A. Prescher, M. J. Hangauer and C. R. Bertozzi, *J. Am. Chem. Soc.*, 2007, **129**, 8400.
6 K. E. Beatty and D. A. Tirrell, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 5995.
7 A. S. Raghavan and H. C. Hang, *Drug Discovery Today*, 2009, **14**, 178.
8 B. F. Cravatt, A. T. Wright and J. W. Kozarich, *Annu. Rev. Biochem.*, 2008, **77**, 383.
9 S. J. Lord, N. R. Conley, H. L. Lee, S. Y. Nishimura, A. K. Pomerantz, K. A. Willets, Z. Lu, H. Wang, N. Liu, R. Samuel, R. Weber, A. Semyonov, M. He, R. J. Twieg and W. E. Moerner, *ChemPhysChem*, 2009, **10**, 55.
10 S. J. Lord, N. R. Conley, H. L. Lee, R. Samuel, N. Liu, R. J. Twieg and W. E. Moerner, *J. Am. Chem. Soc.*, 2008, **130**, 9204.
11 J. Bouffard, Y. Kim, T. M. Swager, R. Weissleder and S. A. Hilderbrand, *Org. Lett.*, 2008, **10**, 37.
12 J. H. Byun, H. Kim, Y. Kim, I. Mook-Jung, D. J. Kim, W. K. Lee and K. H. Yoo, *Bioorg. Med. Chem. Lett.*, 2008, **18**, 5591.
13 K. G. T. Zhang, Ling Qiu and Yuquan Shen, *Synth. Commun.*, 2006, **36**, 1367.

14 G. Charron, M. M. Zhang, J. S. Yount, J. Wilson, A. S. Raghavan, E. Shamir and H. C. Hang, *J. Am. Chem. Soc.*, 2009, **131**, 4967.
15 H. C. Hang, E. J. Geutjes, G. Grotenbreg, A. M. Pollington, M. J. Bijlmakers and H. L. Ploegh, *J. Am. Chem. Soc.*, 2007, **129**, 2744.
16 R. N. Hannoush and N. Arenas-Ramirez, *ACS Chem. Biol.*, 2009, **4**, 581.
17 F. Shao, R. Weissleder and S. A. Hilderbrand, *Bioconjugate Chem.*, 2008, **19**, 2487.
18 P. Kele, X. Li, M. Link, K. Nagy, A. Herner, K. Lorincz, S. Beni and O. S. Wolfbeis, *Org. Biomol. Chem.*, 2009, **7**, 3486.
19 P. Kele, G. Mezo, D. Achatz and O. S. Wolfbeis, *Angew. Chem., Int. Ed.*, 2009, **48**, 344.

