An expanded palette of fluorogenic HaloTag probes with enhanced contrast for targeted cellular imaging

Sylvestre P.J.T. Bachollet,[a] Yuriy Shpinov,[a] Fanny Broch,[a] Hela Benaissa,[a] Arnaud Gautier,[a,b] Jean-Maurice Mallet[a] and Blaise Dumat[a]*

[a] Laboratoire des biomolécules, LBM, Département de chimie, École normale supérieure, PSL University, Sorbonne Université, CNRS, 75005 Paris, France.
[b] Institut Universitaire de France (IUF), 1 rue Descartes, 75005 Paris

*Corresponding author : blaise.dumat@ens.psl.eu

Abstract

We report the development of HaloTag fluorogens based on dipolar flexible molecular rotor structures. By modulating the electron donating and withdrawing groups, we have tuned the absorption and emission wavelengths to design a palette of fluorogens with emissions spanning the green to red range. The probes were studied in glycerol and in presence of HaloTag and exhibited good fluorogenic properties thanks to a viscosity-sensitive emission. In live-cell confocal imaging, the fluorogens yielded only a very low non-specific signal that enabled wash-free targeted imaging of intracellular organelles and proteins with good contrast.

Keywords: Fluorogenic probes; molecular rotors; HaloTag; Fluorescence imaging

Introduction

Fluorescence imaging has become an invaluable tool to study the structure and dynamics of living systems due to its high sensitivity (down to the single molecule), high temporal and spatial resolution and to its biocompatibility, allowing non-invasive imaging of samples in physiological conditions. Numerous fluorescent molecular probes have been developed over time and organic chemistry offers virtually endless possibilities to tailor fluorophores for each application.[1] Nonetheless, since the discovery of the Green Fluorescent Protein (GFP), fluorescent proteins (FPs) have taken a prominent place in the field of bioimaging thanks to the unparalleled targeting selectivity of genetic encoding. A large variety of FPs has since been developed with emission spanning the whole visible spectrum and optimized optical and biochemical properties paving the way to multicolor and complex imaging experiments.[2] More recently, hybrid chemogenetic systems have appeared, that associate a genetically encoded self-labeling protein tag with a small molecular fluorophore linked either covalently or non-covalently to the protein.[3–7] Such combination benefits from the genetic targeting of FPs and from the structural diversity and versatility of organic chromophores and it offers better spatial and temporal control over the fluorescent labeling compared to fluorescent proteins that are continuously expressed and fluorescent. It may nonetheless suffer from common problems associated with molecular probes, including the risk of off-target binding, yielding non-specific signal, and the need to wash the excess of dye. To alleviate these issues, advanced chemogenetic reporters are built on fluorogenic probes that only become fluorescent when bound to the cognate protein tags.[8–11] Considerable efforts have thus been put in the development of fluorogens with diverse optical properties and on their association with protein tags, in particular the most commonly used SNAP-tag[12–15] and HaloTag.[16–21] The latter is, to date, one the most useful tag for covalent protein labeling with high selectivity and fast reaction kinetics. Its chemically simple and lipophilic chloroalkane ligand also facilitates the synthesis of the dedicated probes as well as their cell permeability.[22]

The HaloTag is derived from a bacterial alkane dehalogenase and was built and optimized (up to the last iteration HaloTag7) to bind xanthene dyes and especially rhodamine derivatives with high reaction rates (k_2 = 1.9 x 10^7 $M^{-1}s^{-1}$ for TMR-Halo at 25°C).[3,23] This has driven a lot of the research on HaloTag probes towards rhodamines and, more particularly, fluorogenic Si-Rhodamines taking advantage of the polarity-sensitive spirocyclization reaction.[19,20,22,24–26] This approach certainly yields highly efficient fluorescent chemogenetic

reporters but has narrowed their chemical diversity. Alternative strategies to develop fluorogenic HaloTag probes have relied on the intrinsic viscosity and/or polarity-sensitive emission of dipolar fluorophores.[16,17,27] The approach was pioneered by Kool and coworkers who developed a dimethylamino-styrylpyridinium dye with a viscosity-sensitive emission that was activated upon reaction with HaloTag thanks to the immobilization inside the protein binding channel. These "molecular rotor" structures allow exploring a much wider chemical space: the fluorescence activation mechanism is applicable to a large variety of dipolar structures with electron-donating and withdrawing groups connected through a flexible conjugated bond and is commonly used to develop biomolecular probes.[28–33] Based on this strategy, we have previously reported a series of red and far-red emitting fluorogenic HaloTag probes (Figure 1A).[21] After reaction with HaloTag, the probes are covalently tethered to the protein and exhibit large enhancements of their fluorescence emissions compared to their free form in water (up to 156-fold). The two most promising fluorogens **Red-Halo2** and **NIR-Halo1** enabled wash-free imaging of the nucleus and actin in live cells by targeting HaloTag-NLS and HaloTag-LifeAct fusion proteins respectively. We nonetheless observed a low nonspecific signal in the cytoplasm that may prove detrimental when imaging low-abundant or cytosolic proteins.

Herein, taking advantage of the versatile design, we report an expanded palette of HaloTag fluorogens designed to cover a wider emission range from green to red and to minimize the non-specific signal. The new set of probes was characterized in vitro by absorption and fluorescence spectroscopies and in cell imaging by confocal microscopy. Some new analyses were also performed on the previously reported **Red-Halo2** and **NIR-Halo1** probes for comparison purposes. The photophysical properties were studied *in vitro* in glycerol and in presence of HaloTag to confirm their viscosity-dependent emission and fluorogenic reaction with the protein tag. The selectivity of the probes in wash-free imaging experiments was then studied in cells and, taking advantage of the superior contrast of the newly designed probes, we applied them to image of a variety of cellular organelles and proteins.

Results and discussion

Design and synthesis. The previously reported Red-Halo and NIR-Halo probes cover an emissive range from red to far-red. In order to cover a lower wavelength range, we have modified **Red-Halo2** to introduce two lesser deficient electron-withdrawing groups, 2-cyanoacetic acid or imidazolinone, to form, respectively, the green and yellow emitting derivatives **CCVJ-Halo** and **Y-Halo** (Figure 1B). 2-Cyanoacetic acid gives the CCVJ fluorophore which is a classical molecular rotor with a green emission used as a fluorogenic protein probe and presents a readily available carboxylic acid function for conjugation with the HaloTag ligand.[13,34,35] CCVJ was already coupled to HaloTag but with a different ligand unit which does not readily make the probe fluorogenic upon reaction and was instead used to detect protein aggregation.[27] Imidazolinone the electron withdrawing group found in the GFP chromophore and, in aminated derivatives, it was reported to afford yellow-emitting compounds.[36,37]

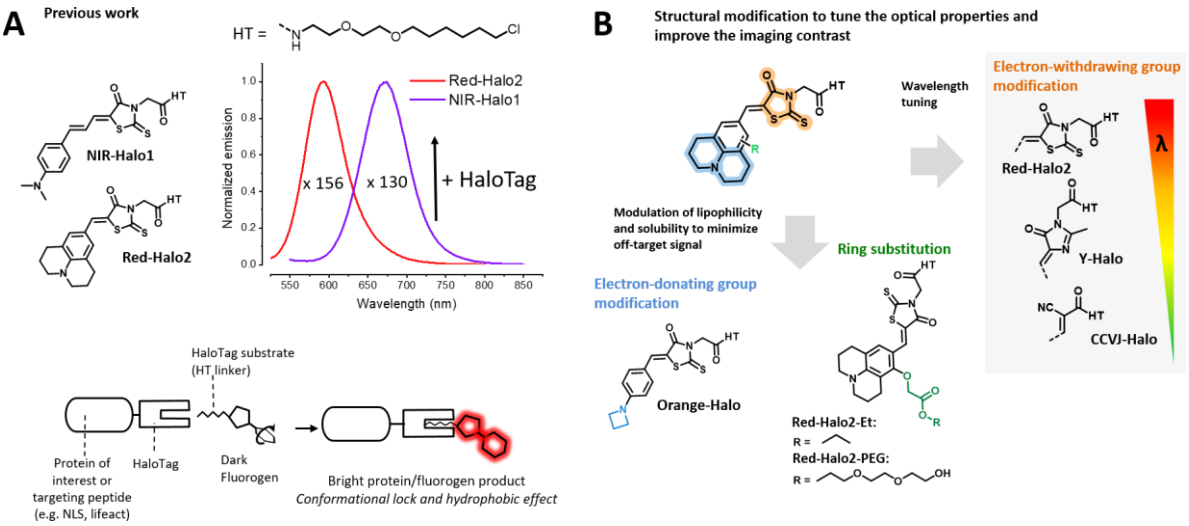

Figure 1. Design and structures of the fluorogenic HaloTag probes.
(A) structures of two of the previously reported probes with their fluorescence emission spectra and fluorescence enhancement upon reaction with HaloTag and general schematic description of the fluorogenic reaction with HaloTag. (B) Structural modifications and structures of the new probes.

In addition to the emission color-tuning, the second goal was to minimize the unspecific signal observed with **Red-Halo2** and **NIR-Halo1** due to the partial activation and/or accumulation of the fluorogens in cytoplasmic organelles. Factors governing the non-specific binding of a dye to intracellular components are difficult to predict and depend in part on the net charge (*e.g*. positively charged compounds binding to mitochondria) and on the lipophilic/hydrophilic balance. The Halo dyes are neutral structures with poor water solubility and we hypothesized that their lipophilic character is one of the driving force of the non-specific binding by leading the probes to accumulate into lipid structures such as the endoplasmic reticulum (ER) membrane. We have thus set out to develop analogs of **Red-Halo2** with lower lipophilicity. As a first strategy, we substituted the aniline ring of **Red-Halo2** with an ethyl or a triethyleneglycol chain through an ester coupling to afford **Red-Halo2-Et** or **Red-Halo2-PEG** respectively (Figure 1B). The ethyl ester is expected to induce only a minute decrease in the lipophilicity of **Red-Halo2**, thanks to the polar ester function, while the addition of a PEG chain is known to increase the hydrophilicity. In a second approach, we have introduced an azetidine donating amino group instead of the julolidine (Figure 1). Azetidine was previously reported as an efficient locked amino donating group since the 4-membered cycle strain prevents the formation of a "twisted intramolecular charge transfer" (TICT) state and it may advantageously replace julolidine while being slightly less lipophilic.[20] Azetidine is also less electron-donating than julolidine and yielded the blue-shifted orange-emitting **Orange-Halo**. Using inexpensive building blocks and reagents, the probes can be obtained in straightforward 2 to 4 steps synthetic procedures detailed in the supplementary information (Scheme S1-S4).

Photophysical properties and reaction with HaloTag. With their flexible dipolar structures, all probes display typical molecular rotor features and we have previously evidenced the fluorogenic behavior of **Red-Halo2** and **NIR-Halo1** in presence of proteins.[21] We have nonetheless confirmed the molecular rotor properties (*i.e.* the viscosity-dependent emission) for the whole series of probes (of the two ester derivatives **Red-Halo2-PEG** and **Red-Halo2-Et**, only the latter was studied) by measuring their spectral properties in water and in glycerol (Figure S1 and Table 1). As expected from the rational design, all probes are weakly fluorescent in water but their emission is strongly enhanced (from 28 to 390-fold) in glycerol with quantum yields ranging from 9 to 20 %, in accordance with an increase in viscosity (Table 1). The largest fluorescence enhancement in glycerol is observed for **Orange-Halo** and is due to the combination of a shift in excitation wavelength and of the fluorescence activation: although the azetidine is slightly less electron-donating than the julolidine, **Orange-Halo** is expected to have spectral properties close to those of **Red-Halo2** but its absorption in water is unexpectedly blue-shifted (409 nm). In glycerol however, a large bathochromic shift is observed resulting in an

absorption spectrum more akin to the parent **Red-Halo2** (Figure S1, Table 1). The viscosity-sensitive emission and good fluorescence brightness observed in glycerol confirm the potential of the probes to be used as fluorogenic biomolecular probes and we next studied the reaction of the probes with a GST-HaloTag (GST-HT) protein *in vitro* and the resulting fluorescence properties of the formed complexes (Figure 2 and Table 1).

Table 1. Photophysical properties of the Halo probes in glycerol and in presence of HaloTag.
λ_{abs} and λ_{em}: maximum absorption and emission wavelength, ε: molar absorptivity coefficient, *FWHM*: Full width at Half maximum of the emission spectrum, Φ_F: fluorescence quantum yield, $\varepsilon \cdot \Phi_F$: brightness, k_2: second order reaction rate constant, F/F_0: Fluorescence enhancement factor where F and F_0 are the integrated fluorescence intensities in glycerol or HaloTag and in water respectively.

Molecule	Solvent/ target	λ_{abs} (nm)	ε (10^3 $M^{-1}cm^{-1}$)	λ_{em} (nm)	FWHM (nm)	Φ_F	$\varepsilon \cdot \Phi_F$	k_2[c] (10^3 $M^{-1}s^{-1}$)	F/F_0
CCVJ-Halo	Glycerol	455	33.9	495		0.19	6441		129
	HaloTag[a]	458	31.8	498	54	0.014	445	1.0 ± 0.1	15
Y-Halo	Glycerol	487	26.2	556		0.099	2594		28
	HaloTag[a]	489	34.9	562	50	0.015	524	n.d.	12
Orange-Halo	Glycerol	486	15.2	578		0.14	2128		390
	HaloTag[a]	487	16.2	574	64	0.020	324	0.45 ± 0.03	48
Red-Halo2-Et	Glycerol	520	14.9	595		0.086	1281		32
	HaloTag[a]	504	14.5	588	65	0.050	725	0.29 ± 0.04	32
Red-Halo2-PEG	HaloTag[a]	525	32.7	599	61	0.055	1800	5.3 ± 0.3	22
Red-Halo2[b]	Glycerol	521	22.9	602		0.20	4580		298
	HaloTag[a]	515	21.0	592	60	0.17	3600	1.3 ± 0.2	156
NIR-Halo1[b]	Glycerol	527	27.5	664		0.091	2503		225
	HaloTag[a]	526	25.8	671	68	0.053	1400	1.7 ± 0.04	130

[a]Measurement conditions: 1 µM dye with 1.3 µM GST-HT in pH 7.4 phosphate buffer containing 10 mM Phosphate and 100 mM NaCl. [b]Data taken from ref. [21] except for k_2 values. [c]Average of duplicate experiments.

All probes undergo a fluorogenic reaction reaction with GST-HT, which allowed us to measure the reaction rate constants by following the increase of fluorescence over time (Figure 2A&B). With the exception of **Y-Halo**, the reactions obey a classical pseudo-first-order exponential law and the calculated second order reaction rate constants are comprised between 0.29 10^3 and 5.3 10^3 $M^{-1}s^{-1}$ (Table 1). The slowest reaction rates were observed for **Orange-Halo** and **Red-Halo2-Et** which can be explained in part by solubility issues: the more hydrophilic **Red-Halo2-PEG** displays the fastest reaction rate, an order of magnitude higher than that of its ethyl ester counterpart. The blue-shifted absorption of **Orange-Halo** in water suggests the formation of aggregates which may also explain its slow reaction kinetics. The reaction with **Y-Halo** is almost instantaneous with an immediate sharp increase of fluorescence that is followed by a slower decrease of intensity that stabilizes after a few minutes (Figure 2B). It is possible that **Y-Halo** undergoes a first non-covalent association with the protein yielding a highly fluorescent complex followed by the covalent enzymatic reaction that leads to a compound with lower fluorescence. Alternatively, it may also adopt several conformations with different emissive properties once bound to the protein. Apart from **Y-Halo**, the reaction rates of the probes are slower than that of rhodamine-based HaloTag ligands but they are in the same range or faster than that observed for other reported solvatochromic HaloTag probes[16,18] and for alternative self-labeling protein tags such as SNAP-tag, CLIP-tag and PYP-tag.[4,5,10,12]

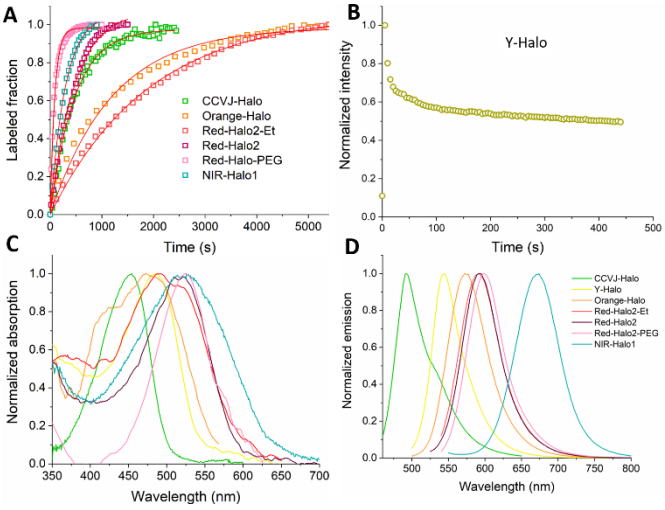

Figure 2. Reaction of the Halo probes with GST-HaloTag.
(A,B) Reaction kinetics of the Halo probes with GST-HT. [dye] = 100 nM, [GST-HT] = 2 µM, T = 298 K. Data was fitted to an exponential rise function (red lines) with the exception of Y-Halo. Normalized absorption (C) and emission (D) of the Halo probes bound to HaloTag. [Dye] = 1 µM [GST-HT] = 1.7 µM.

After reaction with HaloTag, the spectral properties of the bound probes are essentially similar to those in glycerol but with lower fluorogenicities (Figure 2C&D, Table 1). **CCVJ-Halo** and **Y-Halo** display comparable 12 to 15-fold fluorescence activation and while **Red-Halo2-Et, Red-Halo2-PEG** and **Orange-Halo** perform slightly better, the five new probes have lower fluorescence enhancement factors than their parent compound **Red-Halo2** (156-fold), mostly due to lower fluorescence brightnesses. Despite a similar fluorescence activation mechanism, the probes display large differences in fluorogenicities and fluorescence quantum yields that do not reproduce the relative properties observed in glycerol (Table 1). The green and yellow probes in particular are dimmer in HaloTag than in glycerol and the two-step binding reaction of **Y-Halo** may for instance indicate that, of several possible conformations within the protein, the most stable is not necessarily the most emissive. This suggests that optimization of the interaction with HaloTag, through variation of the ligand length and/or mutation of the binding site, may lead to brighter complexes.

Selectivity and contrast in cellular imaging. We next evaluated and compared the selectivity and imaging contrast of the HaloTag fluorogens in wash-free live-cell imaging experiments.

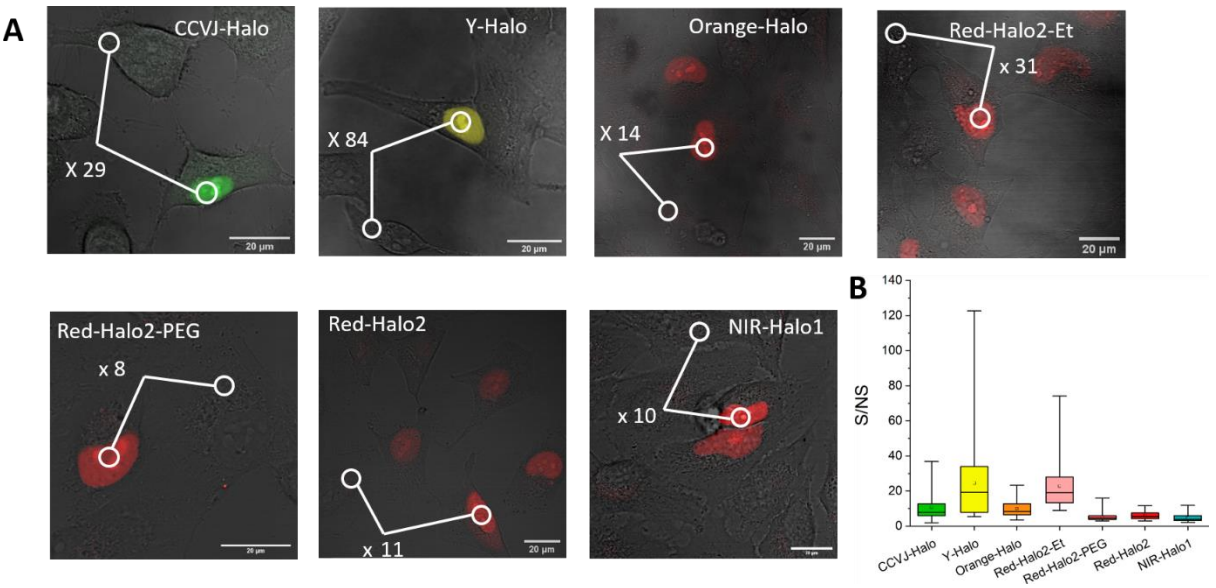

Figure 3. Selectivity and contrast of the Halo probes in cellular imaging.
(A) Live confocal imaging of Hela cells transiently expressing a HaloTag-NLS protein and incubated with 0.5 µM of fluorogenic Halo probe. On each panel is displayed the contrast ratio between the average nuclear intensity and the average cytoplasmic signal measured in a non-transfected cell. λ_{exc} = 514 nm except for CCVJ-Halo (458 nm) and Y-Halo (488 nm). Scale bar: 20 µm (B) Ratio of the specific (S = average intensity in the nucleus) over the non-specific signal (NS = averaged intensity in a cytosolic region of a non-transfected cell). Box and whiskers plot with center lines and squares showing the median and average values for n cells respectively. Box delimits the 25th and 75th percentiles and the whiskers show the max and min values. CCVJ-Halo: n = 64 from four experiments, Y-Halo: n = 91 from three experiments, Orange-Halo: n = 91 from three experiments, Red-Halo2- Et: n = 94 from four experiments, Red-Halo2-PEG: n = 92 from two experiments, Red-Halo2: n = 54 from three experiments, NIR-Halo1: n = 72 from four experiments.

Hela cells transiently expressing the HaloTag-NLS protein were stained with 0.5 µM of Halo probe and were imaged after 30 to 60 minutes without washing steps or medium change. After incubation, the cells showed a good viability with no sign of cytotoxicity and all four new probes were successful in selectively staining the nucleus (Figure 3A). For each fluorogen, we have compared the specific signal (S) in the nuclei with the non-specific signal (NS) in the cytoplasm of an untransfected cell. Untransfected cells were chosen as reference since transfected cells may exhibit some fluorescence in the cytoplasm due to the reaction of the fluorogen with newly synthesized proteins in the cytoplasm. The contrast ratio (S/NS) is given on each panel of figure 3A. For a more precise analysis of the probes selectivity, we have calculated the contrast on a larger set of cells and the distribution of the results is given as a box plot on figure 3B. The specific signal (and thus the related contrast) is highly dependent on the transfection level from one cell to another, which causes a large dispersion of the values, but average and maximum contrast values inform on the relative performance of the probes. Amongst the **Red-Halo2** analogs, the azetidine derivative **Orange-Halo** and **Red-Halo2-Et** give an improved contrast compared to the parent compound **Red-Halo2** but not the more hydrophilic **Red-Halo2-PEG.** These observations are confirmed by the comparison of the fluorescence signal generated by each analog in wild-type Hela cells under identical imaging conditions (Figure S2). **Red-Halo2-PEG** yields the same non-specific cytoplasmic signal as **Red-Halo2** while those of **Orange-Halo** and **Red-Halo2-Et** are respectively 3 and 6-fold lower which is well in line with the relative distribution of the S/NS ratios. The diverging properties of **Red-Halo2-PEG** (higher brightness, faster reaction but lower imaging contrast) and **Red-Halo2-Et** (slower reaction and higher contrast) show that the substitution of the aniline ring is a promising but not easily rationalized strategy to improve the properties of the Halo probes. And, contrary to our initial assumption, the non-specific fluorescence signal in cells is not directly or at least not only linked to the hydrophilic/lipophilic balance of the probes. The green and yellow emitting derivatives, **CCVJ-Halo** and **Y-Halo** also exhibit improved S/NS ratios that reach remarkably high values for the latter. **Y-Halo** yields almost no recordable unspecific signal which, despite its low brightness once bound to HaloTag, makes it a very promising fluorogenic reporter. These results stress the difficulty of designing efficient fluorogenic probes and the necessity to balance *in vitro* and cellular properties: a high fluorescence enhancement factor *in vitro* is an important figure of merit for a fluorogen, yet it does not always translate into a high contrast in cellular imaging. Here, despite its superior optical properties measured in cuvette, **Red-Halo2** is also the most prone to off-target activation in cells yielding a higher non-specific signal. On the other hand, the new probes, with the exception of **Red-Halo2-PEG**, exhibit improved contrast values that are not directly correlated to the relative brightness or fluorescence enhancement values.

Targeted subcellular imaging. In order to take advantage of their increased contrast, we have used the palette of novel HaloTag probes to image of variety of cellular components in addition to the nuclear labeling discussed above (Figure 4 and Figure S3).

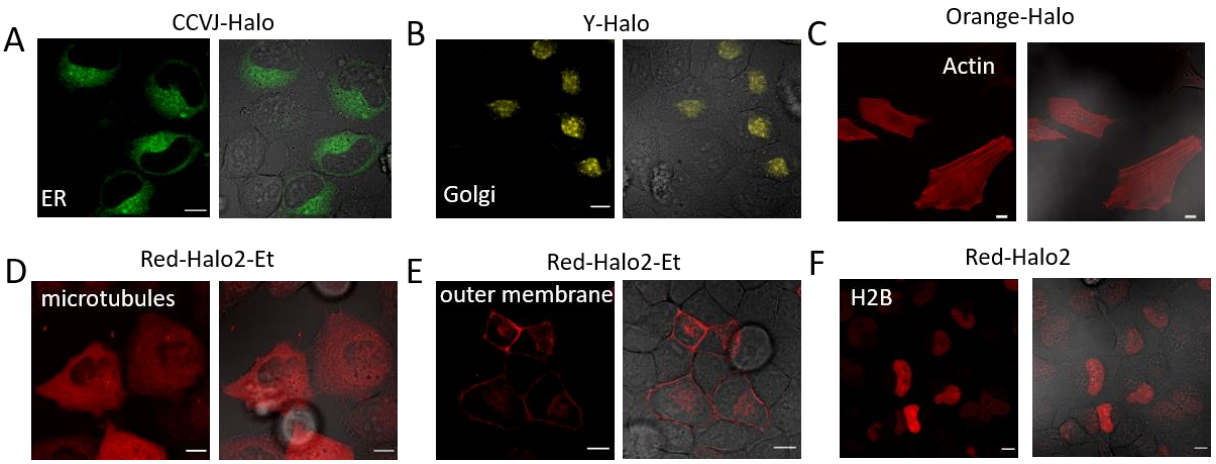

Figure 4. Targeted organelles and proteins imaging.
Live wash-free confocal imaging of Hela cells transiently expressing H2B-HaloTag (nucleus), MAP4-HaloTag (microtubules), PGDFR-HaloTag (external membrane), ER-HaloTag (endoplasmic reticulum), HaloTag-Lifeact (actin) or Golgi-HaloTag (Golgi apparatus) and incubated with 0.5 µM of CCVJ-Halo (A), Y-Halo (B) or Orange-Halo (C) Red-Halo2-Et (D,E) and Red-Halo2 (F). Each panel diplays the fluorescence image (left) and the merged fluorescence and brightfield images (right). Non-transfected cells are visible on the merged images to evidence the selective labeling. λ_{exc} = 514 nm except for CCVJ-Halo (458 nm) and Y-Halo (488 nm) Scale bar: 10 µm.

The parent compound **Red-Halo2** was efficient again to image the nucleus via the targeting of Histone 2B or the cytoskeleton (microtubules) but, in addition to provide a wider range of excitation and emission wavelength, the new probes, thanks to their improved selectivity, enabled the imaging of additional cellular targets, including cytoplasmic organelles. The new fluorogens proved successful in imaging the endoplasmic reticulum (ER), the golgi apparatus, H2B, microtubules, the outer membrane and actin.

Multicolor imaging. CCVJ-Halo, **Y-Halo** and **Orange-Halo** display absorption spectra centered on 458 nm, 489 nm and 487 nm respectively, which allows for an optimal excitation with the 458 and 488 nm Argon laser lines commonly available on microscopes. The **Red-Halo2** derivatives and **NIR-Halo1** can be efficiently excited with the 488 or 514 nm laser lines. Due to their flexible structures that increase the number of available vibrational energy levels, the Halo probes inherently display broad emission peaks. The more flexible structure **NIR-Halo1** displays the broadest peak, with a FWHM of 68 nm and the emission peaks of the **Red-Halo2** analogs have comparable, albeit slightly narrower, breadth (Table 1). Although considerable progress has been made in the analysis and spectral deconvolution of multicolor images, these large emission ranges may render their discrimination from other fluorophores more difficult in multiplexed experiments. On the other hand, the green and yellow derivatives, **CCVJ-Halo** and **Y-Halo**, exhibit narrower emission peaks with FWHM of 54 and 50 nm that could make them more easily applicable in multicolor imaging. As an example, we have successfully combined **Y-Halo** and Mitotracker Deep Red FM in Hela cells expressing Halo-NLS (Figure 5). Both probes can be selectively excited and their emission collected without any cross-talk.

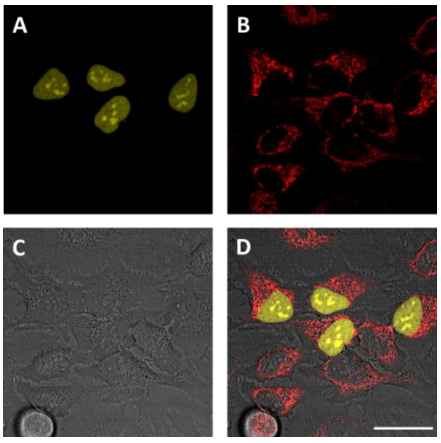

Figure 5. Multicolor imaging.
Live confocal imaging of Hela cells expressing HaloTag-NLS and incubated with 100 nM of Mitotracker Deep Red FM and 0.5 µM of Y-Halo. (A) yellow channel λ_{exc} = 488, collection: 495 – 600 nm, (B) red channel λ_{exc} = 633, collection: 640 – 799 nm, (C) transmission and (D) composite image. Scale bar: 30 µm.

Conclusion

The fluorogenic HaloTag probes reported here enrich a palette of hybrid chemogenetic reporters that covers the green to far-red emission range. They open new possibilities of combination in multicolor imaging, and offer new insight into the design of molecular rotor-based fluorogenic HaloTag probes in order to improve reaction rates and the imaging contrast. Recent reports suggest that HaloTag may be mutated to better fit solvatochromic or molecular rotor probes.[18,38] In parallel to rational molecular design, engineering of the HaloTag protein may thus be envisioned to improve the association with the fluorogens and reach higher quantum yields and faster reaction kinetics.

Experimental procedures

Probes synthesis and characterization. Detailed synthetic procedures and chemical analyses are available in the supplementary information.

Photophysical properties with HaloTag. The fluorogenic dyes were dissolved in DMSO at a stock concentration of 0.5 mM. The interaction of the probes with Halotag was assessed by incubating the dye at 1 µM (0.4 µL of stock solution) concentration with 1.3 µM (ca. 6 µL of commercial stock solution) of protein in a 200 µL working volume of pH 7.4 Phosphate buffer (10 mM phosphate with 100 mM NaCl). Fluorescence spectra were recorded at regular time intervals until completion of the reaction. The different photophysical properties of the protein-bound probes were then measured.

Kinetics study of the labeling reactions. The second-order rate constants of the reactions were calculated by monitoring the fluorescence intensity during the reaction of the probes with a large excess of protein to assume a pseudo first-order kinetics law according to the following procedure.
GST-HT (2 µM) was added to a 50 µL solution of Halo probe (100 nM) in pH 7.4 Phosphate buffer (10 mM Phosphate, 100 mM NaCl). The fluorescence intensity (excitation and emission at the maximum absorption and emission wavelengths of each probe) was then recorded over time until completion of the reaction with a 5 s to 20 s step depending on the probe. The second order rate constants were then calculated according to a previously reported model:[7] the fluorescence was converted to the labeled fraction according to the following equation:

$$Labeled\ fraction = (F-F_0)/(F_{max}-F_0)$$

Where F_{max} and F_0 are the maximum and initial fluorescence intensity respectively. Using Origin 2019 software, the data was fitted to the following equation to calculate the pseudo first-order rate constant k_{obs}:

$$Labeled\ fraction = 1- exp(-k_{obs}t)$$

The second order rate constant k_2 was then obtained by dividing k_{obs} with the protein concentration.

Molecular Biology. Detailed construction of the plasmids used in this study are described in the supplementary information.

Confocal microscopy. Hela cells were grown overnight on 8-well polymer µslides from Ibidi (#1.5 polymer coverslip, tissue culture treated) at 20k to 50k cells/well in 300 µL of MEM culture medium (Gibco) supplemented with 10% fcs, sodium pyruvate and non-essential amino acids. Cells were then transfected with the desired plasmid using Fugene 6 (Promega Corp.) according to the manufacturer's protocol (200 ng of DNA per well with a 3:1 fugene to DNA ratio). After 24 hours, the medium was changed and replaced with DMEM (no phenol red) and the cells were incubated 30-60 minutes with the dyes and imaged live on a Zeiss LSM710

laser scanning confocal microscope equipped with a Plan apochromat 40X/1.4 NA objective. Images were acquired using Zen 2009 software and then processed using ImageJ.

Multicolor imaging with mitotracker Deep Red FM and Y-Halo. Cells were grown according to the protocol above and then labeled Mitotracker Deep Red FM according to the manufacturer protocol. Briefly, Mitotracker was diluted at 100 nM in DMEM (no phenol red) and the cells were incubated for 30 min with the resulting solution. The medium was changed to pure DMEM and the cells were then incubated with **Y-Halo** according to the protocol above. For imaging conditions, see the caption of Figure 5.

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Supplementary information

Supplementary figures, materials and methods, chemical synthesis and molecular biology procedures are available in supplementary information.

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