

Optimized FRET Pairs and Quantification Approaches To Detect the Activation of Aurora Kinase A at Mitosis

Giulia Bertolin,[†] Florian Sizaie,[†] Claire Déméautis,^{†,||} Catherine Chapuis,[†] Fabienne Mérola,[‡] Marie Erard,[‡] and Marc Tramier^{*,†,§,||}

[†]Univ Rennes, CNRS, IGDR (Genetics and Development Institute of Rennes), UMR 6290, F-35000 Rennes, France

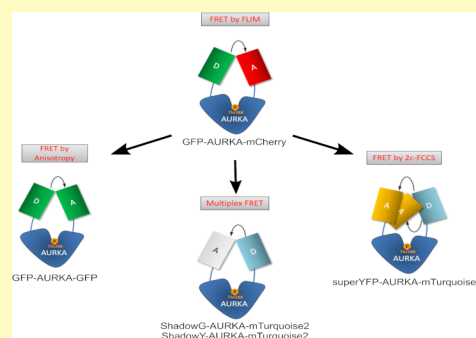
[‡]Univ Paris Sud, CNRS, LCP (Laboratoire de Chimie Physique), UMR 800, F-91400 Orsay, France

[§]Univ Rennes, BIOSIT, UMS CNRS 3480, US INSERM 018, F-35000 Rennes, France

S Supporting Information

ABSTRACT: Genetically encoded Förster's Resonance Energy Transfer (FRET) biosensors are indispensable tools to sense the spatiotemporal dynamics of signal transduction pathways. Investigating the crosstalk between different signaling pathways is becoming increasingly important to follow cell development and fate programs. To this end, FRET biosensors must be optimized to monitor multiple biochemical activities simultaneously and in single cells. In addition, their sensitivity must be increased to follow their activation even when the abundance of the biosensor is low. We describe here the development of a second generation of Aurora kinase A/AURKA biosensors. First, we adapt the original AURKA biosensor—GFP-AURKA-mCherry—to multiplex FRET by using dark acceptors as ShadowG or ShadowY. Then, we use the novel superYFP acceptor protein to measure FRET by 2-color Fluorescence Cross-Correlation Spectroscopy, in cytosolic regions where the abundance of AURKA is extremely low and undetectable with the original AURKA biosensor. These results pave the way to the use of FRET biosensors to follow AURKA activation in conjunction with substrate-based activity biosensors. In addition, they open up the possibility of tracking the activation of small pools of AURKA and its interaction with novel substrates, which would otherwise remain undetectable with classical biochemical approaches.

KEYWORDS: Aurora kinase A/AURKA, mitosis, fluorescence anisotropy, multiplex FRET, superYFP, 2-color FCCS



Genetically encoded Förster's Resonance Energy Transfer (FRET) biosensors have revolutionized our knowledge of signal transduction pathways in the cell. The capability of sensing the activation of kinases, the activity of caspases, or the transport of second messengers as Ca^{2+} or cAMP opened up the possibility of following biochemical reactions in real time and with a spatiotemporal resolution.^{1–3} The novel frontier of these probes consists of combining two or more FRET biosensors at a time to unravel the interdependence of signal transduction pathways, an approach known as multiplex FRET.^{4–10}

AURKA is a serine/threonine kinase with multiple functions in the cell. AURKA was first described to be a mitotic protein with multiple partners and playing key roles in centrosome maturation, in the regulation of mitotic timing, and in building and stabilizing the mitotic spindle.¹¹ Nonetheless, it is now becoming increasingly clear that AURKA has several roles outside of mitosis, such as favoring neurite outgrowth,¹² transcriptional activity through the MYC promoter,¹³ and mitochondrial homeostasis.^{14,15} In this light, it is still unknown whether mitotic and nonmitotic functions of AURKA are made possible by a single, recycling pool of the kinase or by different pools at specific subcellular locations. Given the fact that the

abundance of AURKA is regulated throughout the cell cycle, sensitive techniques are required to decipher the role of AURKA at selected compartments and especially when the abundance of the protein is low as in the G1 phase.

We previously engineered an AURKA FRET biosensor where the donor–acceptor FRET pair flanks the entire AURKA sequence. This new strategy for FRET biosensors allowed us to replace the endogenous kinase in mammalian cells, and to report on its activation at centrosomes both at mitosis and during the G1 phase.¹⁶ We here develop two independent strategies to ameliorate this probe: first, a single-color AURKA biosensor for multiplex FRET and, second, a method to estimate FRET efficiency in regions where the abundance of AURKA is low. We show that FRET donor–acceptor pairs with dark acceptors are preferable for engineering multiplex FRET approaches with the AURKA biosensor, rather than fluorescence anisotropy. We also optimize 2-color Fluorescence Correlation Spectroscopy (2c-FCCS), together with an improved donor–acceptor FRET pair, to detect

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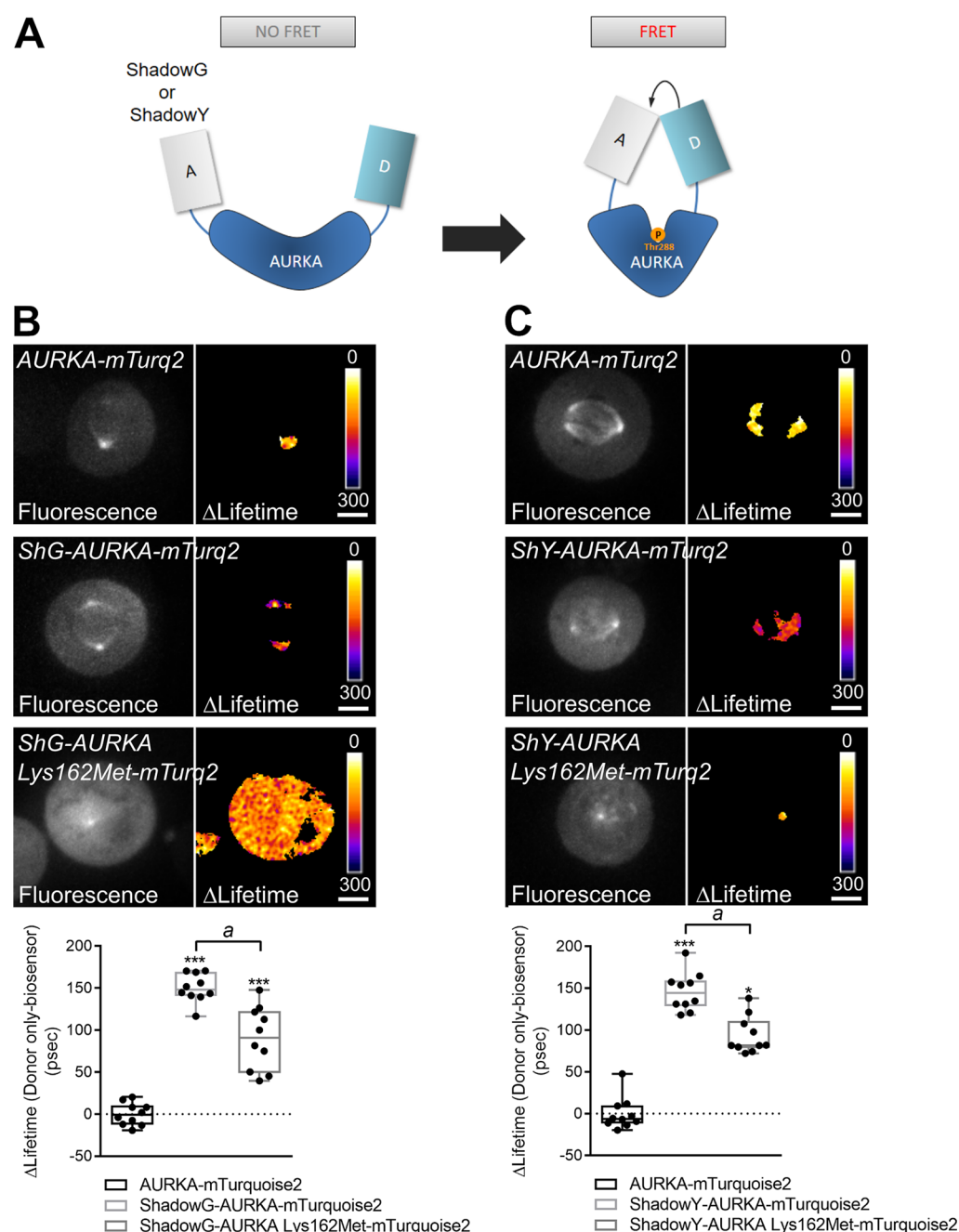


Figure 2. ShadowG and ShadowY are efficient dark acceptors for mTurquoise2 in the AURKA biosensor. (A) Model illustrating the mode of action of the ShadowG-AURKA-mTurquoise2 or the ShadowY-AURKA-mTurquoise2 biosensors. These biosensors switch from an open-to-close conformation upon autophosphorylation of AURKA on Thr288, bringing the donor and the acceptor in vicinity and allowing FRET detection. (B and C) (Upper panels) Representative fluorescence (mTurquoise2 channel) and Δ Lifetime (donor only biosensor) images of U2OS cells expressing the indicated constructs and synchronized at mitosis. (Lower panel) Corresponding Δ Lifetime quantification at the mitotic spindle. ShG: ShadowG; ShY: ShadowY; mTurq2: mTurquoise2. Δ Lifetime values for individual cells are represented as black dots in each boxplot. The bar in boxplots represents the median; whiskers extend from the 10th to the 90th percentiles. $n = 10$ cells per condition of one representative experiment (of three). Scale bar: 10 nm. *** $P < 0.001$ against the "AURKA-mTurquoise2" condition; * $P < 0.05$ compared to the "ShadowG-AURKA-mTurquoise2" condition in B. or * $P < 0.05$ compared to the "ShadowY-AURKA-mTurquoise2" condition in C.

HindIII restriction sites of AURKA-mTurquoise2, respectively. To obtain the mTurquoise2-ShadowG tandem, a tandem containing mTFP1 and ShadowG⁹ was digested with *BglII*/AgeI to replace mTFP1 with mTurquoise2. To obtain the mTurquoise2-ShadowY tandem, a pEGFP-N1 backbone vector containing mTurquoise2 was digested with *BglII*/*Bam*HI to insert ShadowY. To create the mTurquoise2-superYFP tandem, a pEGFP-N1 backbone vector containing a tandem of Aquamarine and superYFP was digested with AgeI/*BglII* to replace Aquamarine with mTurquoise2. To obtain

a vector mTurquoise2 alone, the mTurquoise2-superYFP tandem was digested with *Bam*HI to eliminate the superYFP fluorophore. Cloning reactions were performed with the NEBuilder HiFi DNA Assembly Mix (New England Biolabs) or by T4 DNA ligase (Thermo Fisher Scientific). All cloning reactions were verified on a 3130 XL sequencer (Applied Biosystems). All restriction enzymes were purchased from Thermo Fisher Scientific. The Lys162Met variant for all AURKA biosensors analyzed in the study were obtained from the corresponding wild-type constructs by QuikChange site-directed

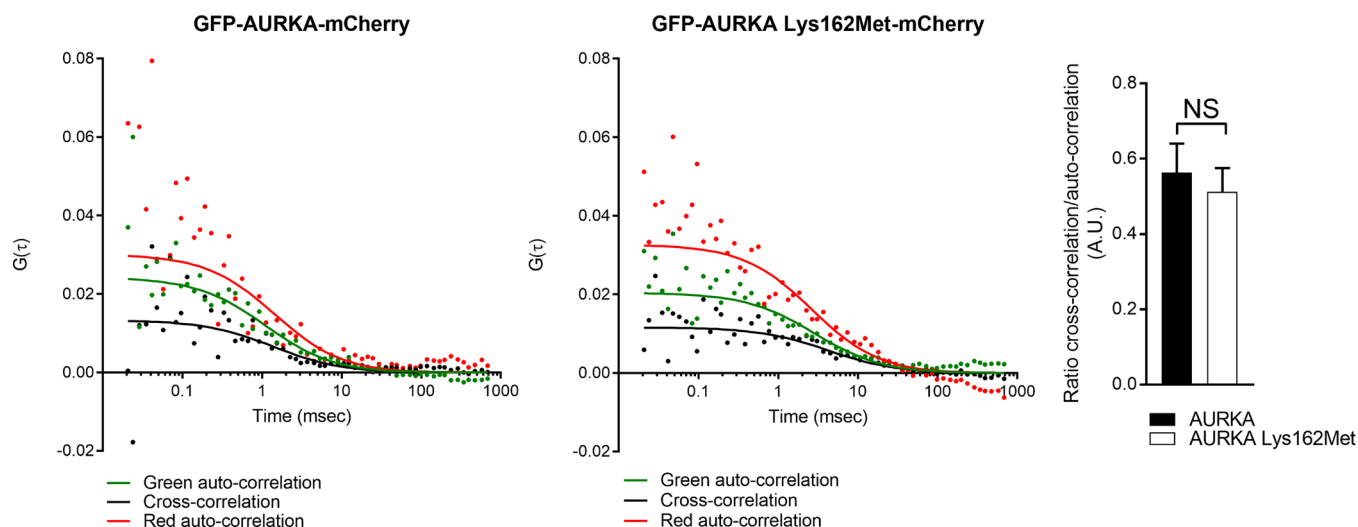


Figure 3. GFP-AURKA-mCherry biosensor does not perform FRET by 2c-FCCS. Green and red autocorrelation curves, together with the respective cross-correlation curves issued from one representative U2OS cell expressing GFP-AURKA-mCherry (left panel) or GFP-AURKA Lys162Met-mCherry (middle panel) and synchronized at mitosis. Measurements were taken in the cytosol; one independent point per condition is shown. Experimental values obtained for each curve are represented as dots. Time is expressed in ms. (Right panel) Ratio of the cross-correlation/green autocorrelation values for GFP-AURKA-mCherry (black bar) and GFP-AURKA Lys162Met-mCherry (white bar). Each n represents the average of three independent points per cell at 0.01 ms; $n = 10$ cells per condition of one representative experiment (of three). NS: not significant.

mutagenesis (Stratagene) with the following primers: 5'-CAAGTTT-ATTCTGGCTCTTATGGTGTATTTAAAGCTCAGCT-3' (sense) and 5'-AGCTGAGCTTTAAATAACACCATAAGAG-CCAGAATAAAGCTTG-3' (antisense).

Cell Culture and Synchronization Procedures. U2OS cells free from mycoplasma were purchased from American Type Culture Collection (ATCC, HTB-96) and were grown in Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich) supplemented with 10% fetal bovine serum (Life Technologies, Thermo Fisher Scientific), 1% L-glutamine (Life Technologies, Thermo Fisher Scientific), and 1% penicillin–streptomycin (Life Technologies, Thermo Fisher Scientific). The generation of GFP-AURKA, GFP-AURKA-mCherry, and GFP-AURKA Lys 162Met-mCherry stable cell lines was previously described.¹⁶ GFP-AURKA-GFP and GFP-AURKA Lys162Met-GFP stable clones cells were generated by transfecting U2OS cells with X-tremeGENE HP transfection reagent (Roche), following the manufacturer's indications. Stable clones were selected in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, 1% penicillin–streptomycin, and 500 μ g/mL Geneticin (Invivogen). Stable and transient transfections were performed with X-tremeGENE HP transfection reagent (Roche) or with Lipofectamine 2000 (Life Technologies, Thermo Fisher Scientific), according to the manufacturer's instructions. For live microscopy, cells were incubated in phenol red-free Leibovitz's L-15 medium (Thermo Fisher Scientific), supplemented with 20% fetal bovine serum, 1% L-glutamine, and 1% penicillin–streptomycin. Mitotic cells were obtained after synchronization at the G2/M transition with 100 ng/mL nocodazole (Sigma-Aldrich) for 16 h. Cells were washed twice with 1X PBS and incubated with prewarmed imaging medium for 30 min to reach metaphase before performing fluorescence anisotropy, FRET/FLIM or 2c-FCCS analyses. All live microscopy experiments were performed at 37 °C in Nunc Lab-Tek II Chamber slides (Thermo Fisher Scientific).

Fluorescence Anisotropy Microscopy. Fluorescence anisotropy analyses were performed on a SP8 (Leica) inverted confocal microscope with a 63X oil immersion objective (NA 1.4) and using the Leica Acquisition Suite (LAS)-AF software. As the excitation light coming out of the laser is naturally polarized, an analyzer integrated in the Leica emission filter wheel was placed in the emission pathway before the pinhole. The analyzer rotates from a parallel to a perpendicular orientation to perform a sequential acquisition of the two corresponding polarized images. Images of GFP polarization were

acquired using a 488 nm argon laser, and emission was selected between 500 and 550 nm. Image analysis was performed using the Fiji (ImageJ) software, and fluorescence anisotropy was calculated as in,¹⁷ using the equation $r = (I_{\parallel} - I_{\perp}) / (I_{\parallel} + 2I_{\perp})$, where r is the fluorescence anisotropy, $I_{\parallel}$ and $I_{\perp}$ are the parallel and the perpendicular polarizations, and $I_{\parallel} + 2I_{\perp}$ is the total polarization-independent emission.

FLIM Microscopy. FLIM analyses were performed in the time domain with a time-gated custom-built setup as described in ref 16 and driven by the Inscoper hardware (Inscoper). Briefly, cells were excited at 440 ± 10 nm and at 480 ± 10 nm using a white light laser, and emission was selected using a band-pass filter of 483/35 nm and of 525/50 nm for mTurquoise2 and GFP, respectively. The fluorescence lifetime was calculated with five sequential temporal gates of 2.2 ns each. The mean pixel-by-pixel lifetime was calculated using the following equation: $\langle \tau \rangle = \sum \Delta t_i \cdot I_i / \sum I_i$ where Δt_i is the delay time of the i th image acquired following a laser pulse, and I is the pixel-by-pixel fluorescence intensity in each image. Lifetime measurements and online calculations were performed with the Inscoper software (Inscoper). The pixel-by-pixel lifetime was calculated only when the fluorescence intensity was above 3000 gray levels. To calculate the Δ lifetime, the mean lifetime of the cells expressing the donor alone (AURKA-mTurquoise2) was calculated for each independent experiment. This mean lifetime value was then used to normalize data in all the analyzed conditions.

Two-Color Fluorescence Cross-Correlation Spectroscopy. 2c-FCCS analyses were carried out on a Leica a SP8 (Leica) inverted confocal microscope with a 63X water objective with a correcting ring (NA 1.2), combined with a PicoQuant time-correlated single photon counting (TCSPC) module and a Fluorescence Lifetime Correlation Spectroscopy (FLCS) integration (PicoHarp, PicoQuant). For the GFP/mCherry donor–acceptor pair the following laser sources were used: a pulsed one at 470 nm and a continuous one at 561 nm, while for the mTurquoise2/superYFP pair we used a pulsed laser source at 440 nm and a continuous one at 514 nm. Fluorescence emission was collected simultaneously using two avalanche photodiode detectors (APD and Tau-SPAD, PicoQuant). The two emission channels used were 500–550 nm and 581–654 nm for GFP/mCherry and 467–499 nm and 525–565 nm for mTurquoise2/superYFP. 2c-FCCS measurements were carried out with the Symphotime software (PicoQuant), integrated into the LAS-AF software (Leica). For each cell, the fluctuation of the fluorescence intensity for both donor–

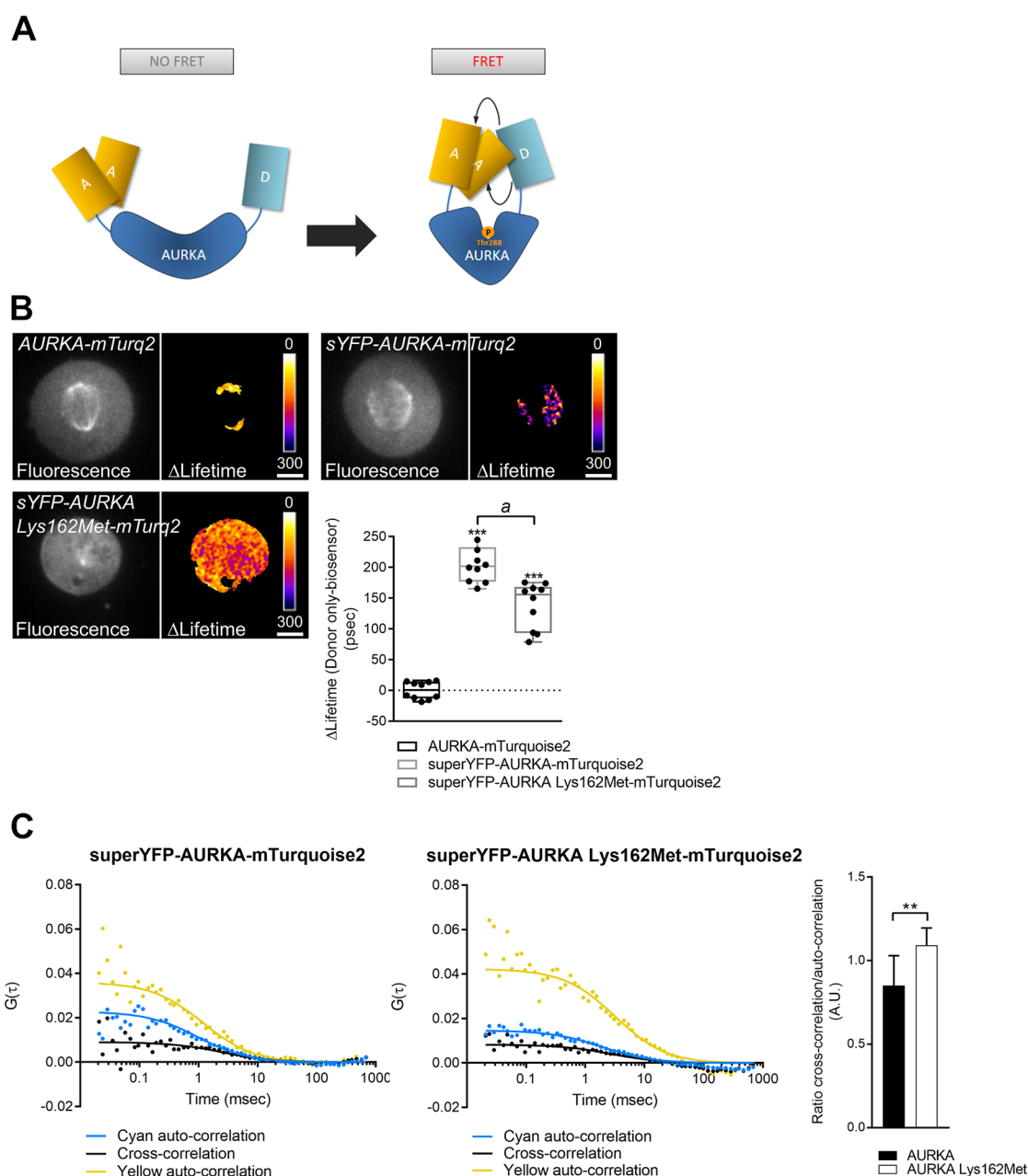


Figure 4. superYFP coupled to mTurquoise2 ameliorates FRET/FLIM detection and allows FRET detection by 2c-FCCS. (A) Model illustrating the mode of action of the superYFP-AURKA-mTurquoise2 biosensor. It switches from an open-to-close conformation upon autophosphorylation of AURKA on Thr288, and allowing FRET measurements. Of note, superYFP is a dimeric acceptor. (B) Representative fluorescence (mTurquoise2 channel) and Δ Lifetime (donor only biosensor) images of U2OS cells expressing the indicated constructs and synchronized at mitosis, together with the corresponding Δ Lifetime quantification at the mitotic spindle. sYFP: superYFP. mTurq2: mTurquoise2. Δ Lifetime values for individual cells are represented as black dots in each boxplot. The bar in boxplots represents the median; whiskers extend from the 10th to the 90th percentiles. $n = 10$ cells per condition of one representative experiment (of three). Scale bar: $10\ \mu\text{m}$. (C) Cyan and yellow autocorrelation curves, together with the respective cross-correlation curves issued from one representative U2OS cell expressing superYFP-AURKA-mTurquoise2 (left panel) or superYFP-AURKA Lys162Met-mTurquoise2 (middle panel) and synchronized at mitosis. Measurements were taken in the cytosol; one independent point per condition is shown. Experimental values obtained for each curve are represented as dots. Time is expressed in ms. (Right panel) Ratio of the cross-correlation/cyan autocorrelation values for superYFP-AURKA-mTurquoise2 (black bar) and superYFP-AURKA Lys162Met-mTurquoise2 (white bar). Each n represents the average of three independent points per cell at 0.01 ms; $n = 10$ cells per condition of one representative experiment (of three) *** $P < 0.001$ against the “AURKA-mTurquoise2” condition (B), ** $P < 0.01$ against the “AURKA” condition (C); ^a $P < 0.001$ compared to the ‘superYFP-AURKA-mTurquoise2’ condition (B).

acceptor pairs (green/red or cyan/yellow) in the confocal volume was analyzed by monitoring three independent points in the cytosol. These points were analyzed sequentially and in nonphotobleaching conditions for 30 s each. Autocorrelation and cross-correlation curves were reconstructed from the fluorescence decay with the Symphotime software and the lifetime integration as previously reported,^{21,22} with

the Symphotime software. Data were fit using the 3D diffusion model, with one diffusing species.^{21,22} The ratio between cross-correlation and green (for GFP/mCherry) or cyan (for mTurquoise2/superYFP) autocorrelation was used to estimate FRET efficiency.

Statistical Analyses. One-way ANOVA and the Dunnett’s method were used to compare the effects of the transfected

AURKA plasmids on GFP fluorescence anisotropy (Figure 1C). One-way ANOVA and the Tukey's method were used to compare the effect of the transfected vectors on fluorescence lifetime (Figure 2B; Supplementary Figure 1A). One-way ANOVA on ranks and the Kruskal–Wallis method were used to compare the effect of the transfected vectors on the fluorescence lifetime (Figures 2C, 4B). The Student's *t* test was used to compare the effect of a GFP monomer and a tandem dimer on fluorescence anisotropy (Figure 1B) or to compare the effects of superYFP on the lifetime of mTurquoise2 (Supplementary Figure 1B); the Mann–Whitney test was used to compare the cross-correlation/autocorrelation ratio between normal and Lys162Met AURKA (Figures 3 and 4C).

RESULTS AND DISCUSSION

Homo-FRET within the AURKA biosensor is undetectable by fluorescence anisotropy microscopy. We previously generated an AURKA FRET biosensor (GFP-AURKA-mCherry) reporting on the autophosphorylation of the protein kinase AURKA on Thr288.¹⁶ Biochemical studies reported that this post-translational modification primes the kinase for activation toward a given substrate.^{23–25} In the perspective of using our FRET biosensor in a multiplex FRET context, we modified the GFP-AURKA-mCherry biosensor to create a single-color FRET sensor to detect homo-FRET. We replaced the mCherry acceptor protein located at the carboxy terminus of the original AURKA biosensor with an Enhanced Green Fluorescent Protein (mEGFP), thereby creating a GFP-AURKA-GFP biosensor (Figure 1A). Changes in the conformation of GFP-AURKA-GFP were analyzed by following the fluorescence anisotropy of the biosensor, obtained by fluorescence anisotropy microscopy.¹⁷ With this approach, decreased anisotropy would correspond to FRET within the biosensor.

We synchronized U2OS cells at mitosis to benefit from a cell cycle phase in which AURKA is active and highly abundant at the mitotic spindle (Nikonova et al., 2013), and where the GFP-AURKA-mCherry biosensor displays significant hetero-FRET.¹⁶ To establish the maximum decrease in anisotropy detectable with our fluorophore pair, we compared a GFP monomer and a tandem dimer. For both constructs, we imaged the parallel and perpendicular components of the emitted fluorescence. This was performed by exciting the sample with a polarized laser, followed by the detection through an analyzer in a confocal microscope setup. Similarly to what previously described, the dimer displays a decreased anisotropy (mean anisotropy: 0.22) compared to the GFP monomer (mean anisotropy: 0.30) in mitotic cells, thus indicating the homotransfer between the two GFP fluorophores harboring different orientations (Figure 1B).¹⁷ This is consistent with the fact that the rotation period of the GFP fluorophore is slower than the fluorescence lifetime.^{26,27} We then evaluated the anisotropy of GFP-AURKA, of the biosensor GFP-AURKA-GFP, and of the kinase-dead version of the biosensor, GFP-AURKA Lys162Met-GFP,¹⁶ on the mitotic spindle. As previously described,¹⁶ the Lys162Met variant of AURKA induces mitotic spindle abnormalities such as monopolar or multipolar spindles. The anisotropy of GFP-AURKA was not different from the one of the GFP monomer, indicating that the presence of the kinase does not perturb the anisotropy of GFP *per se* (Figure 1C).

However, we did not detect any significant difference between the anisotropy of GFP-AURKA and of the biosensor, both in the activated and in the kinase-dead version (Figure 1C). Although fluorescence anisotropy was recently shown to

be a suitable approach for FRET biosensors,¹⁰ relative differences in anisotropy between active and inactive biosensors are often very low. In the case of the AURKA biosensor, the difference between the active and the inactive forms of the biosensor was not significant, with the GFP-AURKA-GFP showing a slightly increased anisotropy rather than a decreased one. This could be due to the clustering of the GFP-AURKA-GFP biosensor at the spindle. Indeed, the high mechanical tension taking place at this compartment at mitosis could keep the fluorophores in a stable conformation, altering the rotational motility of GFP. Alternatively, GFP rotation could also be perturbed by the presence of selected interactors of AURKA at the spindle, which could maintain the biosensor in a fixed conformation. Last, our results could also indicate that the two GFP fluorophores are in a parallel conformation, making differences in anisotropy undetectable and preventing the use of this approach for multiplex FRET in our biosensor setup.

Dark acceptors ShadowG and ShadowY allow for single-color FRET/FLIM measurements. In the perspective of optimizing a multiplex FRET strategy for the AURKA biosensor, we then explored whether dark acceptors as ShadowG¹⁸ or ShadowY¹⁹ could be used as efficient alternatives to fluorescence anisotropy to this end. These proteins were previously demonstrated to be nonfluorescent variants of the Yellow Fluorescent Protein (YFP) preventing spectral bleedthrough of the first acceptor in the second donor channel, thereby resulting in a suitable strategy for multiplex FRET design. As shown in the original reports, they also behave as good acceptors in FRET/FLIM pairs for mGFP or mClover, respectively. These properties of ShadowG and ShadowY allow for multicolor imaging and FRET/FLIM studies with other fluorescent proteins, and we previously took advantage of these properties of ShadowG for the setup of multiplex FRET analyses. In particular, ShadowG turned out to be an efficient acceptor for the cyan fluorescent protein mTFP1 as well,⁹ and we used the mTFP1-ShadowG donor–acceptor pair in conjunction with a second one constituted by the Large Stoke Shift fluorescent protein mOrange (LSSmOrange) and mKate2. This combination of fluorophores used in FRET biosensors allowed us to record two biochemical activities simultaneously.^{9,28}

In light of these previous data, we first monitored the effect of using ShadowG and ShadowY as dark acceptors for the cyan donor protein mTurquoise2. We observed a decrease in the lifetime of the donor of ~500 ps when ShadowG was present in a tandem construct and of ~800 ps when ShadowY was used (Supplementary Figure 1), demonstrating that both fluorophores are excellent acceptors for mTurquoise2. We then replaced the GFP-mCherry donor–acceptor pair of the original AURKA biosensor with mTurquoise2 and ShadowG or ShadowY, thereby creating ShadowG-AURKA-mTurquoise2 or ShadowY-AURKA-mTurquoise2 (Figure 2B). We then tested the conformational changes of these two biosensors by FRET/FLIM in U2OS cells synchronized at mitosis, by calculating the net difference in the lifetime (Δ Lifetime) between the donor-only construct (AURKA-mTurquoise2) and ShadowG-AURKA-mTurquoise2 or ShadowY-AURKA-mTurquoise2 at the mitotic spindle. Δ Lifetime was used to standardize the decrease in lifetime due to FRET, as lifetime variation is the measured parameter directly dependent on the standard deviation of the experimental setup used (50 ps with our setup¹⁶). We measured a mean

Δ Lifetime of ~ 150 ps for both biosensors (Figure 2B–C), which was similar to the decrease observed for the original GFP-AURKA-mCherry one.¹⁶ With the same approach, we also tested the impact of kinase-dead AURKA on FRET efficiencies. Δ Lifetime values for both biosensors carrying the Lys162Met mutation were half compared to their wild-type counterparts, indicating lower FRET efficiencies when AURKA is catalytically inactive. However, it must be noted that the difference in lifetime between normal and kinase-dead AURKA biosensors with dark acceptors (~ 50 ps) was less remarkable than what was observed with the GFP/mCherry AURKA biosensor (~ 120 ps), where the GFP-AURKA Lys162Met-mCherry lifetime was comparable to the one of the donor alone.¹⁶

Our results show that the replacement of the donor–acceptor pair from GFP-mCherry to mTurquoise2-ShadowG or ShadowY maintains FRET efficiency. In addition, both dark biosensors show a lowered FRET efficiency when the kinase is catalytically dead, although this difference is less pronounced than in the original GFP-AURKA-mCherry biosensor. It should also be noted that the performance of a FRET biosensor cannot be predicted from tandem constructs. In spite of the significantly better performance of the ShadowY-mTurquoise2 tandem compared to the ShadowG-mTurquoise2 one (Supplementary Figure 1), the corresponding AURKA biosensors show an identical response (Figure 2B and C). Therefore, two additional parameters should be kept into consideration: first, the nature of the protein probed within the biosensor and, second, the possibility that the conformational changes of this protein fall within the Förster's radius of the donor–acceptor pair or not.

However, the use of biosensors containing dark acceptors should be considered as a suitable alternative to fluorescence anisotropy for the design of multiplex FRET experiments with the AURKA kinase biosensor. In this light, cumulating a biosensor of AURKA containing a dark acceptor with a substrate-based biosensor of the kinase flanked by the LSSmOrange/mKate2 donor–acceptor pair could allow the simultaneous monitoring of AURKA activation by autophosphorylation on Thr288 and its activity toward a substrate, on the same cell and with a spatiotemporal resolution.

GFP-AURKA-mCherry biosensor is not suitable to estimate FRET by two-color FCCS. It is known that AURKA is a multifunctional kinase with several subcellular locations, with both mitotic and nonmitotic functions. While mitotic AURKA is highly abundant in cells,¹¹ the low abundance of nonmitotic pools of AURKA often represents a limitation in exploring their function. Our current approach based on FRET/FLIM allows analysis of the activation of the kinase only where it is particularly abundant (i.e., the centrosome, the mitotic spindle, or mitochondria),^{16,14} possibly excluding from the analysis other cytosolic pools of AURKA too scarce to be analyzed with this microscopy technique. Therefore, we turned to two-color Fluorescence Cross-Correlation Spectroscopy (2c-FCCS) to estimate FRET in regions of the cytoplasm where the abundance of AURKA is low and the number of photons is insufficient to estimate the activation of AURKA by fluorescence lifetime.

In 2c-FCCS, the fluctuating signal intensities of the red and the green channels in and out of a confocal volume are monitored in real time, and their autocorrelation function is computed together with a cross-correlation function between the two channels. Besides these measurements, we used the

temporal correlation of each fluorophore excited by different lasers (pulsed or continuous) to remove the spectral bleed-through of GFP into the mCherry channel. This was done following a previously published strategy^{21,22} based on Fluorescence Lifetime Correlation Spectroscopy (FLCS).²⁹ We excited GFP with a pulsed laser and mCherry with a continuous laser. We then gave a specific weight to each photon in the green and in the red channel using pulsed or continuous calculated lifetime filters. In order to eliminate cross-talk effects between GFP and mCherry, these weights were used to build the autocorrelation curves for each fluorophore and the cross-correlation curve. Applying this strategy to two separate molecules of GFP and mCherry gives a null cross-correlation, thereby eliminating potential artifacts in 2c-FCCS experiments due to spectral bleed-through.^{21,22} In 2c-FCCS, the presence of FRET is indicated by a simultaneous decrease of the amplitude of the cross-correlation curve and the increased amplitude of the green autocorrelation curve.^{30,31} This allows use of 2c-FCCS to estimate FRET in codiffusing molecules, as it is the case for FRET biosensors.

With this approach, we estimated the green and red autocorrelation curves of the original AURKA biosensor (GFP-AURKA-mCherry) and of the kinase-dead GFP-AURKA Lys 162Met-mCherry variant in cells synchronized at mitosis. For each cell analyzed, we measured three randomly chosen points in the cytosol to estimate FRET efficiency in this compartment. We did not detect any significant difference between active and kinase-dead AURKA when we compared the ratio of the amplitudes of the red or green autocorrelation curves with the amplitude of the cross-correlation curve (Figure 3). This was in apparent contradiction with previous data acquired in cells where the cytosol was strong enough to perform FRET/FLIM analyses,¹⁶ and where we detected FRET with the GFP-AURKA-mCherry biosensor. Therefore, we concluded that the ratio between the cross-correlation and the green autocorrelation is not sufficiently robust to detect changes due to FRET in the original AURKA biosensor.

As FRET cannot be estimated by 2c-FCCS using the original AURKA biosensor, it is possible that mCherry represents a limiting factor for the measurement of FRET via 2c-FCCS, due to its longer dark state compared to the one of EGFP-like proteins,³² and its lower brightness (15.84 for mCherry vs 33.6 for mEGFP; www.fpbases.org). Changes in the donor–acceptor fluorescent pair should then be envisaged to adapt the AURKA biosensor to 2c-FCCS, choosing an acceptor with a higher brightness to ameliorate the robustness of the amplitude of the different correlation curves.

Replacing the donor–acceptor pair is mandatory to adapt the AURKA biosensor to two-color FCCS. To find a suitable donor–acceptor pair to adapt the AURKA biosensor to 2c-FCCS, we first searched for a bright acceptor. We decided to replace the usual acceptors of cyan fluorescent proteins Citrine/Venus (brightness of approximately 69; www.fpbases.org), by superYFP, a novel dimeric yellow fluorescent protein with high brightness based on dLanYFP (the dLanYFP brightness is 112.5; www.fpbases.org). superYFP is a dimer of dLanYFP, with a 12 amino acids linker between the first and the second monomer of dLanYFP replacing the last seven amino acids of the first monomer and the first four amino acids of the second one (Supplementary Figure 2A). The full characterization of the physicochemical properties of superYFP will be described elsewhere. We coupled superYFP to mTurquoise2, as this appeared to be an efficient donor

fluorophore in the dark versions of the AURKA biosensor (Figure 2). We then tested the capacity of mTurquoise2 to act as a donor of energy for superYFP in a tandem construct. mTurquoise2 displayed a Δ Lifetime of ~ 600 ps when superYFP was present in U2OS cells synchronized at mitosis (Supplementary Figure 2B), indicating that this donor–acceptor couple is suitable for FRET/FLIM analyses.

In light of these data, we then engineered the superYFP-AURKA-mTurquoise2 biosensor (Figure 4A). To validate this new biosensor, we evaluated its conformational changes at the mitotic spindle by FRET/FLIM. Again, we compared the Δ Lifetime between the donor-only construct AURKA-mTurquoise2 and superYFP-AURKA-mTurquoise2, and we observed a mean Δ Lifetime of ~ 210 ps in the presence of the acceptor (Figure 4B). Unlike the GFP-AURKA-mCherry original biosensor, the presence of the AURKA Lys162Met mutation did not abolish FRET efficiency, as observed for the variants of the AURKA biosensor containing dark acceptors. The differences in Förster's radius (R_0) for the mTurquoise/superYFP pair (59 Å) compared to GFP/mCherry (51 Å;³³) could explain the behavior of the two biosensors. Applying the FRET equation with the usual value $\kappa^2 = 2/3$, the estimated distance between donor and acceptor is 83 Å for the normal, active AURKA and more than 97 Å for the kinase-dead. Using the same distances for the superYFP-AURKA-mTurquoise2 active and dead biosensors, we calculated lifetime values (2.97 ns for the active form; 3.18 ns for the kinase-dead) compatible with what we measured experimentally (3.15 ns for the active; 3.25 ns for the kinase-dead), taking 3.35 ns as the lifetime of the donor alone. It should be noted that the time-gated detection of our system does not yield an unequivocal absolute lifetime value for mTurquoise2 (theoretical lifetime of mTurquoise2: 4 ns;³⁴ lifetime measured with our system: 3.35 ns). This comes from the fact that we do not adjust the decay with exponential models, but we directly calculate the temporal mean of the decay function. This temporal mean and the lifetime are equal when the function exhibits a monoexponential decay, and for an infinite integral calculation. This is the case for GFP, showing a lifetime of 2.5 ns when measured with an 11-ns time window. However, in the case of mTurquoise2—which has a lifetime of around 4 ns—the calculation corresponds to a finite integral when measured with an 11-ns time window. We previously reported that the expression of finite integrals for the mean lifetime can be corrected, although this is a time-consuming procedure incompatible with real-time imaging.³⁵ We provided evidence that this correction is dispensable for FRET, as the differences between donor-only and biosensors using noncorrected mean lifetime calculations are maintained whatever the choice of fluorescent protein pairs.⁹ Since this allows for real-time imaging, we here choose to use the direct calculation of the mean lifetime even if the absolute value of mTurquoise2 is not reached. Taken together, the difference between active and kinase-dead form is the main parameter to be taken into account to estimate the quality of any AURKA biosensor. In this light, the difference in the mTurquoise/superYFP biosensors is 100 ps, whereas the one observed using the GFP/mCherry biosensors is 130 ps.¹⁶ This indicates that when FRET is calculated by FLIM, this new biosensor does not exhibit better performance.

However, we used this version of the AURKA biosensor for 2c-FCCS, potentially benefiting from the greater brightness of superYFP in the computing of the cross-correlation curve. We

calculated the cyan and yellow autocorrelation curves of superYFP-AURKA-mTurquoise2 and of superYFP-AURKA Lys162Met-mTurquoise2 in U2OS cells synchronized at mitosis (Figure 4C). As done previously for the GFP-AURKA-mCherry biosensor, we measured three independent points per cell, chosen in a random manner in the cytosol. We observed a simultaneous increase of the cyan autocorrelation curve and a decrease of the cross-correlation curve in cells expressing the superYFP-AURKA-mTurquoise2 biosensor when compared to the kinase-dead one. The ratio between the cross-correlation and the cyan autocorrelation was lower in cells expressing the active biosensor than in cells expressing the kinase-dead one (Figure 4C, right panel). We also noticed that the amplitude of the autocorrelation curve of superYFP is higher than the one of mTurquoise2. We reasoned that this could be linked to some photophysical properties of superYFP, induced by the laser power used for monitoring its diffusion. These photophysical properties could have the same time scale as the diffusion. If so, we could not discriminate between the two processes, and the photophysical effect would lead to an increase in the amplitude when using a conventional diffusion model. To verify this, we monitored the amplitude of the autocorrelation curve of superYFP after modifying the power of the 514 nm laser. Given that the 2c-FCCS measurements were done with a laser power of $\sim 1\%$ (Figure 4C), we chose four different laser powers for comparison: 0.5%, 1%, 3%, and 5%. We did not observe any significant alteration in the yellow amplitude, which undergoes a 20% increase at most when switching the 514 nm laser from 0.5% to 1% and then remains constant between 1%, 3%, and 5% (Supplementary Figure 3). The fact that there is no significant change in the amplitude of the yellow autocorrelation curve when changing the laser power suggests that a modification of the photophysical behavior of superYFP may not be responsible for the observed higher amplitude. Another possibility is that the high amplitude of the yellow autocorrelation curve in the 2c-FCCS analyses reflects a lower concentration of the yellow moiety in comparison to the cyan one. This would be indicative of a slower maturation of superYFP compared to mTurquoise2, as superYFP derives from dLanYFP. Taken together, the variations in amplitude of the cyan autocorrelation and of the cross-correlation curves meet the requirements for FRET within the superYFP-AURKA-mTurquoise2 biosensor. Although correlation analyses were proven to be a useful approach to characterize single-molecule FRET,^{36,37} we here report for the first time the use of 2c-FCCS to monitor FRET within a genetically encoded biosensor. Last, this new version of the AURKA biosensor containing an mTurquoise/superYFP donor–acceptor FRET pair is a suitable tool to investigate the activation of discrete pools of AURKA with low abundance and with high spatiotemporal resolution.

CONCLUSIONS

Here we propose several improvements to the original AURKA FRET biosensor, in terms of both changing the donor–acceptor FRET pair for multiplex analyses or exploring new approaches to estimate FRET efficiency. It might be useful to point out that although fluorescence anisotropy was successfully used to follow multiplex FRET among PKA, ERK, and cAMP,¹⁰ this is clearly not a suitable approach for the AURKA biosensor. The results here described indicate that there is no “golden rule” for FRET biosensors, and that the capacity of

every biosensor to give a satisfactory response with a particular methodology approach must be carefully evaluated.

Changing the donor–acceptor FRET pairs was mandatory to cumulate the analysis of the conformational changes of AURKA and of its enzymatic activity toward a particular substrate. Given the fact that AURKA is a multifunctional kinase involved in several biochemical pathways,¹¹ designing a strategy for multiplex FRET is a first, mandatory step to start understanding how AURKA autoactivates and interacts with its wide range of substrates. The spatiotemporal resolution AURKA activation and its concomitant interaction with a substrate, or more at a time, is a crucial aspect in the future development of therapeutic strategies in epithelial cancers showing an overexpression of AURKA.

Changes in the way FRET efficiency is calculated, together with improvements in the donor–acceptor FRET pair, were also required to evaluate the activation of AURKA when its abundance is low. 2c-FCCS with the mTurquoise/superYFP pair efficiently responds to this need, allowing monitoring of the activation of AURKA in the cytosol. We previously described that AURKA is activated at the mitochondrion,¹⁴ a compartment where the kinase is abundant enough to perform FRET/FLIM analyses. The mTurquoise/superYFP AURKA FRET biosensor used in conjunction with fluorescent markers for other subcellular compartments could potentially shed light on the activation of AURKA at other locations, where the kinase is less abundant. Such activation could lead to the identification of novel roles of AURKA, together with novel substrates.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acssensors.9b00486](https://doi.org/10.1021/acssensors.9b00486).

Supplementary Figure 1: efficiency of the mTurquoise-ShadowG or ShadowY donor/acceptor FRET pairs; Supplementary Figure 2: amino acidic sequence of superYFP, and efficiency of the mTurquoise-superYFP donor–acceptor FRET pair; Supplementary Figure 3: superYFP G(0) values at different 514 nm laser powers (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +33223235487. E-mail: marc.tramier@univ-rennes1.fr.

ORCID

Marc Tramier: [0000-0001-8200-6446](https://orcid.org/0000-0001-8200-6446)

Present Address

[†]Leica Microsystems SAS, rue du 1er Mai, Nanterre, France

Author Contributions

G.B. designed, performed, and analyzed the experiments, wrote the manuscript, and provided funding. F.S. and C.D. performed and analyzed the experiments. C.C. provided technical help. M.E. and F.M. provided the superYFP coding sequence and relative vectors, shared unpublished data, and provided advice. M.T. conceptualized the study, coordinated the work, and provided funding.

Notes

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