

Three-Dimensional FRET Multiplexing for DNA Quantification with Attomolar Detection Limits

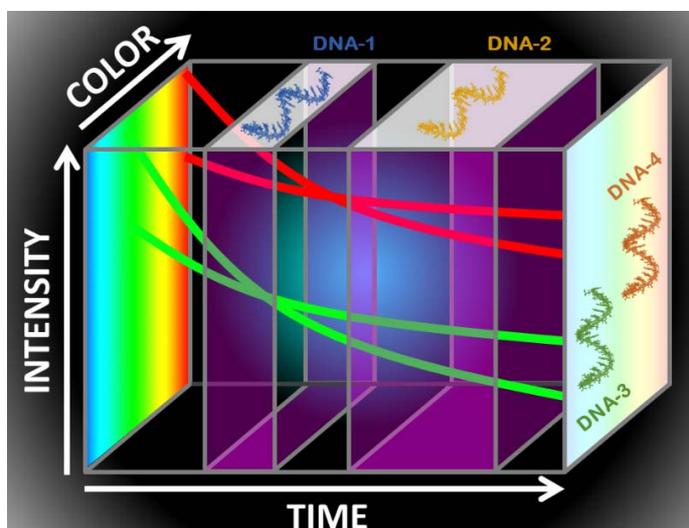
Xue Qiu,⁺ Jiajia Guo,⁺ Jingyue Xu, and Niko Hildebrandt*

NanoBioPhotonics (nanofret.com), Institute for Integrative Biology of the Cell (I2BC), Université Paris-Saclay, Université Paris-Sud, CNRS, CEA, Orsay, France

⁺ These authors contributed equally.

*Corresponding author: niko.hildebrandt@u-psud.fr

ABSTRACT: Photoluminescence (PL) multiplexing usually relies on spectral or temporal separation. Their combination into higher-order multiplexing for biosensing is extremely challenging because the PL intensity is required for target quantification at very low concentrations and the interplay of color, lifetime, and intensity must be carefully adapted. Here, we demonstrate



time-gated Förster resonance energy transfer (TG-FRET) from a long-lifetime Tb-complex to Cy3.5 and Cy5.5 dyes for spectrottemporal multiplexing of four different DNA targets in the same sample by single-color excitation and two-color detection. We used rolling circle amplification (RCA) for high specificity and sensitivity and for placing Tb donors and dye acceptors at controlled distances within the amplified DNA concatemers. This precise distance tuning led to target-specific PL decays of the FRET pairs and simple, separation-free, and higher-order multiplexed quantification of DNA. The RCA-FRET DNA assay could distinguish very homologous target sequences and provided limits of detection down to 40 zeptomoles (300 aM).

Keywords: FRET, multiplexing, fluorescence, time-gating, rolling circle amplification, biosensing, terbium, dyes, DNA, RNA

Understanding the fundamental functions of complicated biochemical systems or diagnosing specific diseases most often require the quantification of many molecules and interactions at extremely low concentrations from a single sample. Photoluminescence detection is ideally suited for multiplexed biosensing, because it is rapid and sensitive and there is an almost unlimited choice of fluorophores that provide a large versatility of photochemical and photophysical properties.^{1,2} Arguably, the most frequently used technique to detect multiple parameters from the same sample is spectral (or color) multiplexing with different fluorophores, such as organic dyes,³⁻⁷ fluorescent proteins,⁸ quantum dots,^{9,10} or lanthanide nanoparticles and complexes.^{11,12} Each fluorophore requires a distinct detection channel and excitation wavelength, unless quantum dots or FRET from one donor to multiple acceptors are used. Spectral overlap, which needs to take into account the entire spectrum of each fluorophore (not only the full-width-at-half-maximum, FWHM), imposes fundamental limits to color multiplexing. Even the spectra of quantum dots (with FWHM of ~30 nm) extend over >100 nm, which limits the spectral range of 400 to 800 nm to approximately four completely distinct signals. Another means of PL multiplexing is to exploit the lifetime of PL. Despite impressive proofs of concept, specific and sensitive biological target quantification has not been accomplished with these approaches.¹³⁻¹⁸ Replacing lifetime fitting by time-gated PL intensity detection in different temporal windows can simplify temporal multiplexing for biosensing but the low nanomolar target concentrations are still too high for many biochemical and biomolecular studies.¹⁹ Although a combination of spectral and temporal multiplexing within a single biosensing technique (higher-order multiplexing) may seem evident, an actual experimental realization is very complicated and such a method is not available. One of the main reasons is the third dimension of PL, namely intensity, which is required for quantifying the analyte. For each biological target or interaction, a three-dimensional PL biosensor must provide a specific and stable spectrotemporal signal combination that is independent of the other targets and the environment and changes its

intensity as a function of target concentration. This objective becomes even more challenging when extremely low concentrations (fM – pM) of various similar targets need to be specifically quantified.

Because sensitive detection of various nucleic acids is among the most important requirements for investigating and understanding fundamental biological processes, a multiplexed and specific quantification of DNA can significantly advance such research. Low abundance of nucleic acids often necessitates their amplification and isothermal methods with specific amplification are versatile alternatives to PCR-based technologies.²⁰ A well-known and highly specific isothermal amplification methods is target-primed rolling circle amplification (RCA), in which a padlock-DNA circularization by ligation over DNA²¹ or RNA²² targets enables the amplification process. In contrast to PCR, target-primed RCA can be applied both *in situ*, *e.g.*, for genotyping individual DNA or mRNA molecules,^{7,23} and in solution, *e.g.*, for DNA²⁴ or micro-RNA (miRNA)²⁵ diagnostics. Two important limitations of photoluminescence (PL)-based RCA are the necessary separation of free fluorescent DNA-probes from those that have hybridized to the RCA product and the reduced capability of multiplexing from a single sample. Nucleic acid staining dyes²⁶ and dye-to-dye FRET probes²⁷ were used to avoid the separation step but still suffered from significant background fluorescence. Single-molecule digital counting techniques were also shown to function without separation but require specific and rather sophisticated experimental setups and procedures.²⁸ Although the long DNA concatemers of RCA have been widely applied for PL signal amplification, the possibility of placing FRET probes at specific and well-controlled distances within the multiple copies of the same DNA sequence has been largely overlooked. As FRET and the involved PL lifetimes are strongly distance dependent,^{29,30} isothermal amplification strategies could be ideally suited for controlled PL lifetime tuning.

Here, we present a nucleic acid detection method that combines the sensitivity and specificity of target-primed RCA with the ratiometric and single-step detection format of time-gated FRET (TG-FRET).³¹ TG-RCA-FRET was used for spectral and temporal multiplexing by precise adjustment of donor-acceptor distances within the amplified DNA sequence at sub-nanometer resolution. The separation-free and background-free assay format accomplished limits of detection (LOD) between 40 and 570 zeptomoles (0.3 to 4.1 fM) for four different DNA targets. We demonstrate the combination of the spectral and temporal dimensions of PL for higher-order multiplexing with the intensity dimension of PL for quantification of the four different DNAs at low femtomolar concentrations from the same sample, using a single excitation source and only two distinct fluorescence colors.

The principle of multiplexed RCA-FRET is shown in **Figure 1a**. First, a DNA target is specifically hybridized by the two ends of a linear probe DNA (padlock DNA) that circularizes upon target recognition. Then, the padlock nick is ligated over the target splint, which makes the target a primer for a polymerase to synthesize complementary DNA around the circular probe DNA. After each round, the previously synthesized DNA strand is displaced, which leads to a long ssDNA concatemer. As the repetitive sequence of this long RCA product is known (by the design of the padlock), FRET-donor (Tb) and FRET-acceptor (Cy3.5 or Cy5.5) DNA probes (cf. **Figure S1** for absorption/emission spectra and FRET parameters) can be designed to hybridize at desired positions within the concatemer and thereby at defined but distinct donor-acceptor distances (R). Due to the strong distance-dependence of the FRET efficiency ($\sim R^{-6}$), different values of R lead to different PL decays of the FRET-quenched donors and FRET-sensitized acceptors (the closer the distance the faster the decay).^{29,30,32} These different PL decays can then be used for straightforward temporal multiplexing by time-gated PL intensity detection.¹⁹ The use of two different acceptors

(Cy3.5 and Cy5.5) for the same Tb donor allows for spectral multiplexing with a single excitation wavelength. In principle, x distinct spectral signals and y distinct temporal signals can be combined to $x \cdot y$ distinct signals in solution or x^y distinct signals if the different RCA products are spatially separated (*e.g.*, *in-situ* imaging). Such higher-order multiplexing has the potential to significantly advance the detection of multiple targets from a single sample. Another advantage is the separation-free format of RCA-FRET because FRET is not possible for Tb and dye DNA probes that are free in solution (not hybridized to the RCA product). Finally, the ratiometric detection of time-gated PL intensities of dye and Tb (**Figure 1b**) leads to very precise (low coefficients of variation) signal quantification.

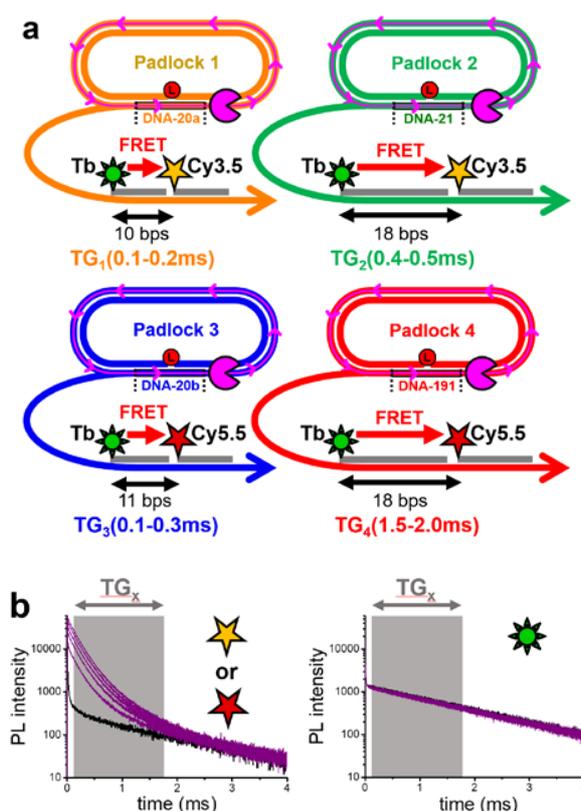


Figure 1. (a) Combining the same Tb donor with two different dye acceptors (Cy3.5 and Cy5.5) and hybridizing the DNA probes at different distances (bps: base pairs) inside the target-specific RCA products, provides four distinguishable signals (two distinct time-gated detection windows TG₁-TG₄ for two different colors) in two detection channels. (b) Ratiometric TG-FRET measures the FRET-ratio of FRET-sensitized dye PL intensity (left) and FRET-quenched Tb PL intensity (right) within a specific time-window after pulsed excitation (to suppress autofluorescence). Black/purple decay curves present signals without/with target. The FRET-sensitized dye-acceptor intensity strongly increases with increasing target concentration, whereas the overall Tb-donor signal is not significantly quenched (because also free Tb DNA-probes in solution emit PL).

As a representative system for multiplexed nucleic acid detection, we selected four different short ssDNAs (DNA-20a, DNA-20b, DNA-21, and DNA-191), which are the DNA-equivalents of the micro-RNAs hsa-mir-20a/20b/21/191, all of which have been found to be related to breast cancer.³³⁻³⁷ Because target-primed RCA via padlock probes was already applied for longer DNA as well as RNA targets,^{7,21-23} our short ssDNA-based detection is in principle transferable to other nucleic acids. To evaluate the sensitivity and versatility of RCA-FRET, we quantified the four DNAs at concentrations between 1 and 250 fM and with two different TG-FRET donor/acceptor pairs (Tb/Cy3.5 and Tb/Cy5.5). All eight RCA-FRET sensors showed a linear signal-over-concentration behavior within the tested concentration range, which allowed for the distinction of even very similar concentrations, and LODs between 40 and 570 zeptomoles (0.3 to 4.1 fM) of DNA (**Figure 2**). For specificity testing, DNA-20a and DNA-20b had only two nucleotide variations, from which one was at the 5'-terminus. All four sensors provided specificity solely to their respective targets without non-specific background signals from the other three DNAs and with increasing FRET signals over a concentration range of more than three orders of magnitude (**Figure S4**).

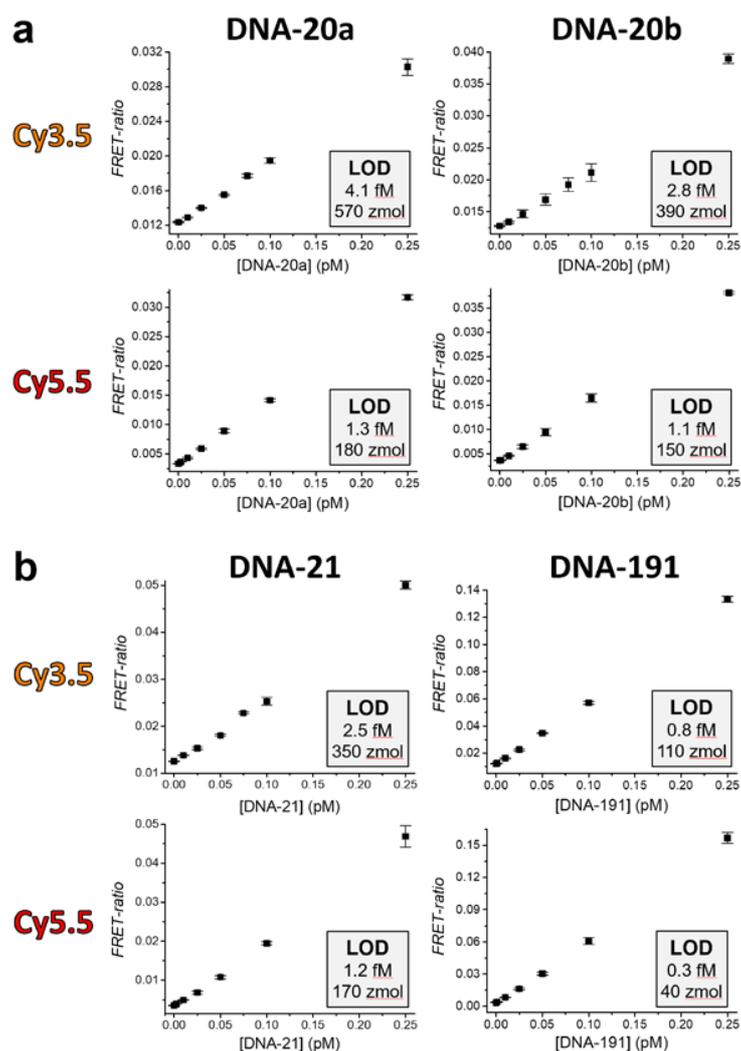


Figure 2. RCA-FRET assay calibration curves for four different DNA targets (see **Table S1** for sequences) with Cy3.5 or Cy5.5 as FRET acceptor (cf. **Figure S2** for PL decay curves that were used to calculate the FRET-ratios in a TG window from 0.1 to 0.9 ms). LODs were 4.1 ± 0.6 fM (Cy3.5) and 1.3 ± 0.2 fM (Cy5.5) for DNA-20a, 2.8 ± 0.4 fM (Cy3.5) and 1.1 ± 0.2 fM (Cy5.5) for DNA-20b, 2.5 ± 0.4 fM (Cy3.5) and 1.2 ± 0.2 fM (Cy5.5) for DNA-21, and 0.8 ± 0.1 fM (Cy3.5) and 0.3 ± 0.1 fM (Cy5.5) for DNA-191 (cf. **Figure S3** for determination of LODs).

Multiplexing from a single sample is highly important to simultaneously measure various targets, to reduce sample volume and reagents, and to increase the measurement throughput. We have previously shown that Tb-to-dye TG-FRET can be used for efficient and sensitive color multiplexing in immunoassays (five targets)³ and miRNA assays (three targets)^{38,39} and for temporal multiplexing of three different nucleic acids.¹⁹ However, neither the combination of these two concepts into higher-order spectrotemporal TG-FRET multiplexing from a single sample, nor

the detection of extremely low concentrations (fM – pM) of biomolecules has been realized. Following the RCA-FRET concept from **Figure 1a**, we designed four different padlocks and six different FRET probes (four Tb probes and two dye probes), which allowed us to place the two different FRET-pairs at distinct distances within the target-specific RCPs. This, in turn, led to distinct PL decay curves (**Figure S5**) due to the different FRET efficiencies. All PL signals were generated by using a single excitation source because the same Tb donor was used in all cases. The DNA-target concentration-dependent FRET-ratios in four distinct time-gated detection windows (**Figure 3a**) were used to calibrate the multiplexed RCA-FRET assay. To test the 4-plex assay, we prepared 10 samples with varying concentrations of the four DNA targets. **Figure 3b** shows that the measured concentrations were in excellent agreement with the actual target concentrations. Notably, all DNA concentrations within the different samples were in a range from 40 to 500 fM and therefore three to four orders of magnitude lower compared to previous temporal multiplexed nucleic acid detection without amplification.¹⁹ This impressive sensitivity improvement clearly shows the combined advantages of RCA and TG-FRET not only for multiplexing but also for the precise quantification of extremely low target concentrations. Similar results were found for a 2-plex assay of DNA-20a and DNA-21 using only the Tb/Cy5.5 FRET-pair (**Figure S6**).

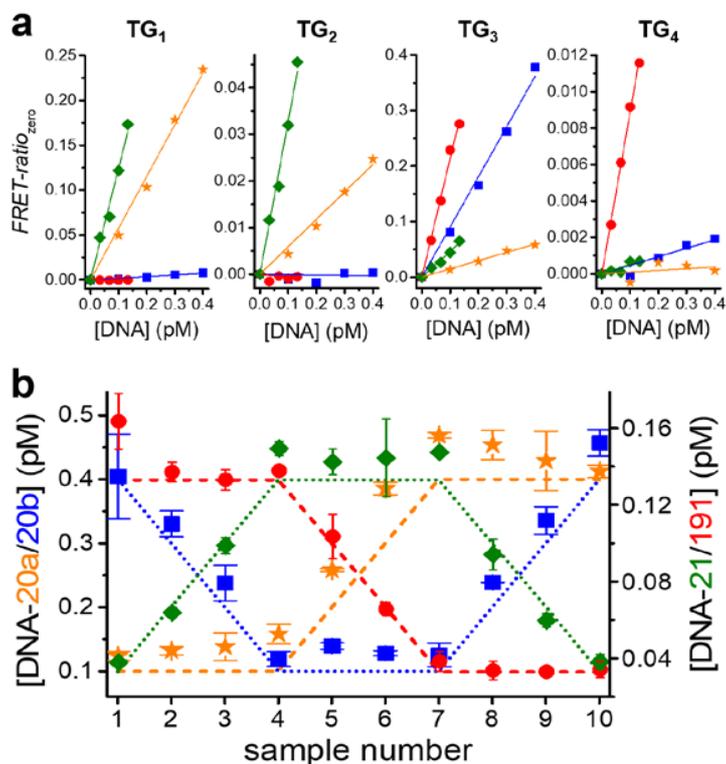


Figure 3. (a) Contributions of the different RCA-FRET signals ($FRET-ratio_{zero}$) to the different time-gated detection windows. The calibration curves (lines) were used to calculate the concentrations for the 4-fold TG-FRET multiplexed assays (see Supporting Information for mathematical treatment). (b) Recovery of varying concentrations of DNA-20a, 20b, 21, and 191 from 10 different samples using spectrottemporal TG-FRET multiplexing. Dotted lines represent known concentrations; data points represent RCA-FRET measurements.

In conclusion, RCA-FRET is the first method that can combine spectral and temporal multiplexing into higher-order multiplexed and highly sensitive detection of nucleic acids. It can be performed by straightforward procedures on commercially available time-resolved fluorescence plate readers and is adaptable to other RCA approaches and probably to any other DNA-amplification strategy that uses hybridization of fluorescent probes. Although our method was applied to liquid samples, target-primed RCA with padlock probes has been frequently applied for *in situ* imaging in cells,^{7,23} where high sensitivity and simultaneous multiplexing is also of utmost importance.⁴⁰ RCA-FRET provided LODs down to 40 zeptomoles (300 aM) of DNA, which presents an improvement of more than four orders of magnitude compared to non-amplified TG-

FRET detection of the same targets.³⁸ Spectrotemporally multiplexed detection (from the same sample) of four different DNA targets in the 40 to 500 fM concentration range was accomplished by using only two different FRET-pairs and one excitation wavelength. These target concentrations were three orders of magnitude lower compared to spectral multiplexing of three DNA targets with three different FRET pairs.³⁸ This first demonstration of spectrotemporal multiplexing quantified only four DNAs by combining two spectral and two temporal components, but the use of TG-FRET with five different acceptors³ and three different time-gated windows¹⁹ would allow for 15-fold multiplexing from a single liquid sample or 125-fold multiplexing for imaging (**Figure S7**). Although such extensions are beyond the current scope, our future research will investigate the experimental limits of spectrotemporal multiplexing and the adaption of RCA-FRET to *in-situ* imaging in cells. The versatility, specificity, and sensitivity of isothermal RCA-FRET for higher-order multiplexed quantification of nucleic acids at extremely low concentrations are ideally suited to advance many fundamental and applied biological studies. Because no other technology can combine such technological versatility with accuracy and simplicity of application, we believe that RCA-FRET will be of high value both in research and diagnostics, and provide new insights about the role of DNA or RNA in biological functions and diseases.

ASSOCIATED CONTENT

Supporting Information

Materials and Methods; Absorption and emission spectra of the RCA-FRET pairs (Figure S1); PL decay curves (of Tb and dyes) for the different DNA targets (Figure S2); Determination of LODs (Figure S3); Dynamic concentration range and high specificity of RCA-FRET (Figure S4); PL decay curves for calibration of spectrotemporal TG-FRET multiplexing (Figure S5); 2-plex spectral multiplexing (Figure S6); Spectrotemporal multiplexing capabilities for 5 colors and 3

time-gates (Figure S7); Sequences and modifications of all DNA probes and targets (Table S1). The Supporting Information is available free of charge on the ACS Publications website.

Acknowledgements

We thank Lumiphore, Inc. for the gift of Lumi4® reagents. This work was supported by the French National Research Agency (ANR project "AMPLIFY"), the Institut Universitaire de France (IUF), the China Scholarship Council (CSC), and the European Commission (Horizon 2020, FET-Open project PROSEQO).

Notes

Université Paris-Sud, CNRS, and Lumiphore have filed a provisional patent application, EP2017061882, on the described method of detecting or quantifying one or multiple nucleic acid targets. X.Q. and N.H. are inventors on this patent application.

REFERENCES

- (1) Hötzer, B.; Medintz, I. L.; Hildebrandt, N.: Fluorescence in Nanobiotechnology: Sophisticated Fluorophores for Novel Applications. *Small* **2012**, *8*, 2297-2326.
- (2) Sapsford, K. E.; Wildt, B.; Mariani, A.; Yeatts, A. B.; Medintz, I. L.: Materials for FRET Analysis: Beyond Traditional Dye-Dye Combinations. In *FRET - Förster Resonance Energy Transfer. From Theory to Applications*; Medintz, I. L., Hildebrandt, N., Eds.; Wiley-VCH: Singapore, 2014.
- (3) Geissler, D.; Stufler, S.; Löhmansröben, H. G.; Hildebrandt, N.: Six-Color Time-Resolved Förster Resonance Energy Transfer for Ultrasensitive Multiplexed Biosensing. *J. Am. Chem. Soc.* **2013**, *135*, 1102-1109.
- (4) Bae, S. W.; Tan, W. H.; Hong, J. I.: Fluorescent dye-doped silica nanoparticles: new tools for bioapplications. *Chem. Commun.* **2012**, *48*, 2270-2282.
- (5) Faulds, K.; Jarvis, R.; Smith, W. E.; Graham, D.; Goodacre, R.: Multiplexed detection of six labelled oligonucleotides using surface enhanced resonance Raman scattering (SERRS). *Analyst* **2008**, *133*, 1505-1512.
- (6) Lin, C. X.; Liu, Y.; Yan, H.: Self-assembled combinatorial encoding nanoarrays for multiplexed biosensing. *Nano Lett.* **2007**, *7*, 507-512.
- (7) Larsson, C.; Grundberg, I.; Soderberg, O.; Nilsson, M.: In situ detection and genotyping of individual mRNA molecules. *Nat. Methods* **2010**, *7*, 395-U81.
- (8) Cai, D.; Cohen, K. B.; Luo, T.; Lichtman, J. W.; Sanes, J. R.: Improved tools for the Brainbow toolbox. *Nat. Methods* **2013**, *10*, 540-+.
- (9) Geissler, D.; Charbonnière, L. J.; Ziessel, R. F.; Butlin, N. G.; Löhmansröben, H. G.; Hildebrandt, N.: Quantum Dot Biosensors for Ultrasensitive Multiplexed Diagnostics. *Angew. Chem. Int. Ed.* **2010**, *49*, 1396-1401.
- (10) Medintz, I. L.; Farrell, D.; Susumu, K.; Trammell, S. A.; Deschamps, J. R.; Brunel, F. M.; Dawson, P. E.; Mattoussi, H.: Multiplex Charge-Transfer Interactions between Quantum Dots and Peptide-Bridged Ruthenium Complexes. *Anal. Chem.* **2009**, *81*, 4831-4839.
- (11) Gorris, H. H.; Wolfbeis, O. S.: Photon-Upconverting Nanoparticles for Optical Encoding and Multiplexing of Cells, Biomolecules, and Microspheres. *Angew. Chem. Int. Ed.* **2013**, *52*, 3584-3600.
- (12) Pershagen, E.; Borbas, K. E.: Multiplex Detection of Enzymatic Activity with Responsive Lanthanide-Based Luminescent Probes. *Angew. Chem. Int. Ed.* **2015**, *54*, 1787-1790.
- (13) Cui, H. H.; Valdez, J. G.; Steinkamp, J. A.; Crissman, H. A.: Fluorescence lifetime-based discrimination and quantification of cellular DNA and RNA with phase-sensitive flow cytometry. *Cytometry Part A* **2003**, *52A*, 46-55.
- (14) Grabolle, M.; Kapusta, P.; Nann, T.; Shu, X.; Ziegler, J.; Resch-Genger, U.: Fluorescence Lifetime Multiplexing with Nanocrystals and Organic Labels. *Anal. Chem.* **2009**, *81*, 7807-7813.

- (15) Hoffmann, K.; Behnke, T.; Drescher, D.; Kneipp, J.; Resch-Genger, U.: Near-Infrared-Emitting Nanoparticles for Lifetime-Based Multiplexed Analysis and Imaging of Living Cells. *ACS Nano* **2013**, *7*, 6674–6684.
- (16) Hoffmann, K.; Behnke, T.; Grabolle, M.; Resch-Genger, U.: Nanoparticle-encapsulated vis- and NIR-emissive fluorophores with different fluorescence decay kinetics for lifetime multiplexing. *Anal. Bioanal. Chem.* **2014**, *406*, 3315–3322.
- (17) Lu, Y. Q.; Lu, J.; Zhao, J. B.; Cusido, J.; Raymo, F. M.; Yuan, J. L.; Yang, S.; Leif, R. C.; Huo, Y. J.; Piper, J. A.; Robinson, J. P.; Goldys, E. M.; Jin, D. Y.: On-the-fly decoding luminescence lifetimes in the microsecond region for lanthanide-encoded suspension arrays. *Nat. Commun.* **2014**, *5*.
- (18) Lu, Y. Q.; Zhao, J. B.; Zhang, R.; Liu, Y. J.; Liu, D. M.; Goldys, E. M.; Yang, X. S.; Xi, P.; Sunna, A.; Lu, J.; Shi, Y.; Leif, R. C.; Huo, Y. J.; Shen, J.; Piper, J. A.; Robinson, J. P.; Jin, D. Y.: Tunable lifetime multiplexing using luminescent nanocrystals. *Nat. Photonics* **2014**, *8*, 33–37.
- (19) Qiu, X.; Guo, J. J.; Jin, Z. W.; Petreto, A.; Medintz, I. L.; Hildebrandt, N.: Multiplexed Nucleic Acid Hybridization Assays Using Single-FRET-Pair Distance-Tuning. *Small* **2017**, *13*.
- (20) Zhao, Y. X.; Chen, F.; Li, Q.; Wang, L. H.; Fan, C. H.: Isothermal Amplification of Nucleic Acids. *Chem. Rev.* **2015**, *115*, 12491–12545.
- (21) Nilsson, M.; Malmgren, H.; Samiotaki, M.; Kwiatkowski, M.; Chowdhary, B. P.; Landegren, U.: Padlock Probes - Circularizing Oligonucleotides for Localized DNA Detection. *Science* **1994**, *265*, 2085–2088.
- (22) Nilsson, M.; Barbany, G.; Antson, D. O.; Gertow, K.; Landegren, U.: Enhanced detection and distinction of RNA by enzymatic probe ligation. *Nat. Biotechnol.* **2000**, *18*, 791–793.
- (23) Larsson, C.; Koch, J.; Nygren, A.; Janssen, G.; Raap, A. K.; Landegren, U.; Nilsson, M.: In situ genotyping individual DNA molecules by target-primed rolling-circle amplification of padlock probes. *Nat. Methods* **2004**, *1*, 227–232.
- (24) Thomas, D. C.; Nardone, G. A.; Randall, S. K.: Amplification of padlock probes for DNA diagnostics by cascade rolling circle amplification or the polymerase chain reaction. *Arch. Pathol. Lab. Med.* **1999**, *123*, 1170–1176.
- (25) Jonstrup, S. P.; Koch, J.; Kjems, J.: A microRNA detection system based on padlock probes and rolling circle amplification. *RNA* **2006**, *12*, 1747–1752.
- (26) Li, Y.; Liang, L.; Zhang, C. Y.: Isothermally Sensitive Detection of Serum Circulating miRNAs for Lung Cancer Diagnosis. *Anal. Chem.* **2013**, *85*, 11174–11179.
- (27) Wu, X. R.; Zhu, S. R.; Huang, P. Y.; Chen, Y. J.: Highly specific quantification of microRNA by coupling probe-rolling circle amplification and Förster resonance energy transfer. *Anal. Biochem.* **2016**, *502*, 16–23.
- (28) Jarvius, J.; Melin, J.; Goransson, J.; Stenberg, J.; Fredriksson, S.; Gonzalez-Rey, C.; Bertilsson, S.; Nilsson, M.: Digital quantification using amplified single-molecule detection. *Nat. Methods* **2006**, *3*, 725–727.
- (29) Hildebrandt, N.: How to Apply FRET: From Experimental Design to Data Analysis. In *FRET - Förster Resonance Energy Transfer. From Theory to Applications*; Medintz, I. L., Hildebrandt, N., Eds.; Wiley-VCH: Weinheim, Germany, 2014; pp 105–164.
- (30) Lakowicz, J. R.: *Principles of fluorescence spectroscopy*; 3rd ed.; Springer: New York, N.Y., 2006.
- (31) Zwiern, J.; Hildebrandt, N.: Time-gated FRET detection for multiplexed biosensing. In *Reviews in Fluorescence 2016*; Geddes, C. D., Ed.; Springer International, 2017.
- (32) Valeur, B.: *Molecular Fluorescence : Principles and Applications*; Wiley-VCH: Weinheim ; New York, 2002.
- (33) Schwarzenbach, H.; Milde-Langosch, K.; Steinbach, B.; Muller, V.; Pantel, K.: Diagnostic potential of PTEN-targeting miR-214 in the blood of breast cancer patients. *Breast Cancer Res. Treat.* **2012**, *134*, 933–941.
- (34) Li, S. F.; Qiang, Q.; Shan, H. T.; Shi, M. K.; Gan, G. M.; Ma, F.; Chen, B. J.: MiR-20a and miR-20b negatively regulate autophagy by targeting RB1CC1/FIP200 in breast cancer cells. *Life Sci.* **2016**, *147*, 143–152.
- (35) Mar-Aguilar, F.; Luna-Aguirre, C. M.; Moreno-Rocha, J. C.; Araiza-Chavez, J.; Trevino, V.; Rodriguez-Padilla, C.; Resendez-Perez, D.: Differential expression of miR-21, miR-125b and miR-191 in breast cancer tissue. *Asia-Pac. J. Clin. Oncol.* **2013**, *9*, 53–59.
- (36) Xie, B. Y.; Ding, Q.; Han, H. J.; Wu, D.: miRCancer: a microRNA-cancer association database constructed by text mining on literature. *Bioinformatics* **2013**, *29*, 638–644.
- (37) Xie, B.; Ding, Q.: <http://mircancer.ecu.edu>. <http://mircancer.ecu.edu>, May 2018.
- (38) Jin, Z. W.; Geissler, D.; Qiu, X.; Wegner, K. D.; Hildebrandt, N.: A Rapid, Amplification-Free, and Sensitive Diagnostic Assay for Single-Step Multiplexed Fluorescence Detection of MicroRNA. *Angew. Chem. Int. Ed.* **2015**, *54*, 10024–10029.
- (39) Qiu, X.; Hildebrandt, N.: Rapid and Multiplexed MicroRNA Diagnostic Assay Using Quantum Dot-Based Förster Resonance Energy Transfer. *ACS Nano* **2015**, *9*, 8449–8457.
- (40) Marx, V.: Probes: FRET sensor design and optimization. *Nat. Methods* **2017**, *14*, 949–953.