Fluorescence imaging using synthetic GFP chromophores

Christopher L. Walker,^a Konstantin A. Lukyanov,^b Ilia V. Yampolsky,^{b,c} Alexander S. Mishin,^b Anna M. Duraj-Thatte,^a Bahareh Azizi,^a Laren M. Tolbert,^a Kyril M. Solntsev^{a,*}

^a *School of Chemistry and Biochemistry, Georgia Institute of Technology, 901 Atlantic Drive, Atlanta, GA 30332-0400, United States*

^b *Institute of Bioorganic Chemistry, Russian Academy of Sciences, Miklukho-Maklaya 16/10, 117997 Moscow, Russia*

^c *Pirogov Russian National Research Medical University, Ostrovitianov 1, Moscow 117997, Russia*

Abstract

Green Fluorescent Protein and related proteins carry chromophores formed within the protein from their own amino acids. Corresponding synthetic compounds are non-fluorescent in solution due to photoinduced isomerization of the benzylideneimidazolidinone core. Restriction of this internal rotation by binding to host molecules leads to pronounced, up to three orders of magnitude, increase of fluorescence intensity. This property allows using GFP chromophore analogs as fluorogenic dyes to detect metal ions, proteins, nucleic acids, and other hosts. For example, RNA aptamer named Spinach, which binds to and activates fluorescence of some GFP chromophores, was proved to be a unique label for live-cell imaging of specific RNAs, endogenous metabolites and target proteins. Chemically locked GFP chromophores are brightly fluorescent and represent potentially useful dyes due to their small size and high water solubility.

Introduction

Fluorescent proteins are widely used for imaging of cellular structures, for elucidation of biochemical trafficking, and for other biochemical/biophysical applications. The most successful of these have been based upon the green fluorescent protein (GFP), green because of an inherent

excited-state proton transfer (ESPT) that is the basis of the fluorescence mechanism. The “heart” of the GFP of is a *p*-hydroxybenzylideneimidazolidinone (Figure 1), in which the hydroxygroup acts as the proton donor in the ESPT. A large color palette of fluorescent proteins have been developed through random mutagenesis, but the applications have been limited because of the restriction to a ca. 27 kD protein which may not be compatible many applications, and for which the reliance on bioavailable aromatic amino acids restrict the diversity of emission properties. This observation has stimulated research on other chromophores (Cros) derived from the arylideneimidazolidine core, using biomimetic methodology to sequester the chromophores and turn on fluorescence through topological non-bonding effects. Such methods hold promise not only in imaging but in elucidating biochemical pathways.

Our groups have worked diligently on the development of new chromophores and new sequestering mechanisms which cause fluorescence to turn on in response to specific recognition mechanisms.^{1,2} This includes protein/chromophore interactions, lipid/chromophores interactions, and chromophores aggregation. Topological and weak-bonding interactions can thus be used as inherent probes for biochemical environments.

Modification of the Cro skeleton, leading to changes in fluorescence intensities, emission wavelengths, and general fluorescence properties is well known.³⁻⁶ For our purposes, we consider the Fluorescence Intensity ratio (FI), as the primary indicator of sequestration. In reality, of course, FI is really a measure of relative decay lifetimes, and we associate increased lifetime with a decrease in decay rates due to minimization of vibrational and rotational modes which otherwise lead to fast internal conversion. In this mini-review we will describe only the applications of the synthetic FP Cros for fluorescence imaging, and some fluorescence studies in solutions which can be potentially applied for imaging. Indeed, numerous classes of various fluorogens are known⁷⁻⁹ which are used for the fluorimetric determination of analytes described in this mini-review. However, the uniqueness of the GFP Cro-based fluorogens is that to this class of compounds is used to detect the extremely broad range of ions and macromolecular hosts as shown in the current paper. The nearest competitors are the derivatives of tetraphenylethylene, the aggregation-induced emission dye.

Aggregation-induced emission

An inhibition of the intramolecular isomerization and torsional motion, i.e. a restriction of the internal rotations (RIR), can be achieved upon temperature decrease or in the solid state. These degrees of freedom are often associated with the fluorescence quenching. A relatively rare effect of fluorescence turn-on upon crystallization is named aggregation-induced emission (AIE).¹⁰ AIE

became an indispensable tool for molecular sensing and imaging¹¹⁻¹³ due to chromophores' higher resistance to photobleaching and huge fluorescence on/off ratio. We have serendipitously discovered that the long chain ethers of parent *p*-hydroxybenzilidenedimethylimidazolinone **1** (but not **1** itself) exhibit AIE (Figure 2a). The color tuning of the resulting emission in the solid-state of **2a-c** is governed by the monomer/excimer ratio in the fluorescence spectra, which in turn is determined by the packing motif of the fluorophores. Later, we have demonstrated that the observed AIE phenomenon can be utilized for the production of fluorescent microcrystals and nanofibers using the same Cros.¹⁴

Since our discovery of the AIE in the GFP Cros, additional related compounds have exhibited this behavior (**3-7**).¹⁵⁻¹⁹ Curiously, in contrast to the long-chain ethers (**2a-c**) and the compounds with polycyclic aromatic substituents (**3a-e**),¹⁵ the alkyl derivatives (**6**)¹⁶ demonstrated very little color tuning with the variation of the alkyl substituent. It is interesting that some Cros (**5a-d**) exhibit AIE in cell membranes not through the RIR mechanism but via exclusion of solvent-solute hydrogen bonding, proposed by Yang to be responsible for fluorescence quenching.²⁰

Ion sensing

We have demonstrated that ultrafast photoisomerization in GFP Cros chromophores can be suppressed by metal ion complexation using an aza-derivative **8a**, for which the phenol moiety in **1** is replaced with a 2-pyridyl moiety.²¹ Dramatic enhancement of fluorescence (FI = 150) in the presence of Zn²⁺ and Cd²⁺ ions was observed compared with other metals (Figure 2b). However, the dissociation constant of the Zn²⁺-**8a** complex (K_d 0.5 mM) showed its moderate stability. The metal binding properties of another bidentate ligand, the synthetic derivative of the blue fluorescent protein chromophore **9**,²² are not studied yet. To increase the sensitivity of GFP-based metal ligands, further development led to creation of tri- and tetradentate ligands (**10-13**).²³⁻²⁵ The latter showed an impressive stability (K_d < 30 nM) and were utilized for Zn²⁺ sensing in vitro.²⁵ We have also modified **8a** converting it into the hydroxyl derivative **8b**, in which the fluorescence and “super” photoacidity are turn-on by metal complexation.²⁶

The only example of using the GFP fluorophores for anion sensing is the compound **14** which turns off its fluorescence selectively in the presence of fluoride ions that hydrolyze Si-O and O-H bonds.²⁷

Chromophores fused to macromolecules and polymers

In order to mimic the natural environment of the protein β -barrel, several approaches were made to fuse the GFP Cros to various macromolecules. The GFP Cro covalently bound to β -cyclodextrin **15**²⁸ showed the formation of a self-inclusion complex, but the FI was only two-fold. Goodson et al utilized a discovered earlier non-linear optical property (two-photon absorption, 2PA) of GFP Cros²⁹ for the amyloid aggregation detection.³⁰ No FI was observed at very low concentrations of the attached dye-peptide system **16**. However, at the same conditions it showed an impressively large 2PA cross section: 540 GM vs. 32 GM for the free Cro. The authors claimed that this jump of 2PA can be used for diagnostic applications in neurodegenerative diseases and might be able to reveal answers to conformational questions about amyloid- β (1–42). Few other GFP-related dyes were also used in 2PA imaging.³¹

Very elegant examples of macromolecular water-soluble GFP Cro – based polymers were recently reported by Zhu et al.^{32,33} The PEG-PNIPAM-Cro diblock copolymer **17** demonstrated a temperature-induced fluorescence enhancement behavior. Indeed, the PNIPAM moiety was responsible for the reversible temperature dependence of the GFP Cro fluorescence which turned on (FI = 8) at temperature above LCST due to the collapse of PNIPAM chain block. This behavior was utilized for *Bacillus thermophilus* bacterial detection (Figure 2c). Another example was the amphiphilic PEG-Cro-PMMA polymer **18** with enhanced fluorescent properties (FI = 24) after self-assembly into micellar aggregates. Both polymers were applied for cell imaging.

Locked chromophores

Introduction of a difluoroboryl group in GFP Cros derivatives proved an important method for creating compounds highly fluorescent in solution.²⁶ Aminated conformationally locked GFP chromophores are red-shifted, highly fluorescent compounds with strong solvatochromic behavior. For instance, **19**³⁴ showed more than 20-fold FI between water and hydrophobic solvents and about 30 nm hypsochromic change in emission maximum. This cell-permeable chromophore rapidly stained the plasma membrane and certain cellular lipid organelles in live and fixed cells without washing out the dye. As with other fluorogens, the constant exchange of the dye allowed for high photostability of labelling. It was possible to achieve further bathochromic shift in the BF₂-locked GFP Cros by locking the rotation of the amino group,³⁴ and introducing additional conjugation as in **20**.³⁴ Chromophore **21**, conformationally-locked by difluoroboryl group and linked to cathepsin inhibitor has been used as an activity-based probe, capable of discerning active form of human cathepsin for in-gel imaging.³⁵

Matchmaking for GFP chromophores. First candidates.

To study GFP Cro complexation in a systematic way and to study the selectivity of fluorescence probes, Chang et al. screened a library of 41 Cro toward 94 biologically relevant random analytes to generate fluorescence response profiles (Figure 2d).³⁶ This practical high-throughput screening created a target-oriented fluorescent library³⁷ of our synthetic Cro. A lot of compounds were found to be promiscuous, i.e. turned on their fluorescence in the presence of various hosts. Some “hits” were then studied in details. We started with simple hosts such as octa-acids³⁸ and cholates.³⁹ The compounds demonstrating the largest FI upon binding in both systems were ortho-substituted derivatives **22** (FI 38 in octa-acids) and **23** (FI 212 in cholates).

Nucleic acid sensing. From DNA modification to binding RNA aptamers

Two main approaches of nucleic acid sensing using the GFP Cro are known in the literature. The first one involves a covalent attachment of Cro to oligonucleotides (conceptually similar to the Cro-macromolecules fusion described earlier), while the second one is based on the optimization of Cro/DNA (RNA) interactions. The compound **24** synthesized by Riedl et al⁴⁰ was used for a facile enzymatic synthesis of oligonucleotide or DNA probes by polymerase-catalyzed primer extension. Upon protein binding a modest FI (2-3.2 times) was detected. Nevertheless, the concept was demonstrated on sequence-specific binding of p53 to dsDNA and on nonspecific binding of single strand binding protein to an oligonucleotide. A straightforward approach of Wenge and Wagenknecht to incorporate the GFP Cro into oligonucleotides using a postsynthetic methodology (**25**)⁴¹ was not spectacular since the observable quantum yields of the GFP chromophore in DNA were too small to be relevant for immediate applications. In contrast, the polycyclic GFP derivative **7**¹⁹ of Ikejiri et al. demonstrated a FI of about 500 in the presence of dsDNA, which is significantly higher than that of ethidium bromide (FI ~10), which is generally employed in dsDNA detection. This compound was also AIE-active.

For today, perhaps the most developed application of synthetic GFP-like Cro is labeling of target RNA using aptamers. This approach was suggested by Jaffrey and colleagues in 2011.⁴² In this work, RNA aptamers which specifically bind some GFP-like Cro were generated. These aptamers were found to strongly (up to 2000-fold) activate fluorescence of the chromophores due to steric constraints of the chromophore isomerization upon binding to the aptamer. The brightest pair consisted of a 98-nucleotide aptamer named Spinach and chromophore DFHBI (**26**), which interact non-covalently with K_d of about 0.5 μ M (Figure 3). Introduction of two fluorine atoms into GFP chromophore resulted in strongly reduced pK_a . **26** is practically nonfluorescent in solution, but

brightly fluoresces green (quantum yield 0.72) in a complex with Spinach. Few other chromophores of different colors (**27-29**) demonstrated similar behavior, but with weaker FI.

Crystallographic studies of Spinach-**26** complex revealed structural basis of their interactions.^{43,44} Spinach RNA forms a bent 11-nm long stick-like structure (Figure 3a). It was found that a G-quadruplex plays a central role in **26** binding. Together with a base triple and an unpaired G, the G-quadruplex form a pocket where **26** adopts coplanar *cis* conformation stabilized by stacking with nucleotide bases and some H-bonds (Figure 3b).

In spite of its utility for certain tasks, Spinach-**26** possesses serious drawbacks which hamper its wide applicability: (i) poor folding efficiency in intracellular condition; (ii) sensitivity to flanking RNA sequences; (iii) low temperature stability (melting temperature T_m 34°C); (iv) strong dependence on Mg^{2+} ions; (v) rather big size. These problems were partially solved in further developed aptamers. For example, site-directed mutagenesis of Spinach resulted in Spinach2 with improved folding and thermostability (T_m 38°C) displaying several-fold increased brightness in live cells compared to parental Spinach.⁴⁵ Fluorescence of both Spinach and Spinach2 was found to be strongly enhanced by inserting it into tRNA that acts as a rigid scaffold.^{42,45} Also, a twofold shorter variant dubbed Baby Spinach was generated using crystal structure to remove unessential parts of Spinach RNA.⁴⁴ Finally, a new approach to select RNA aptamers based on their ability to activate **26** fluorescence inside live bacterial cells was developed.⁴⁶ This method allowed generating a new 49 nucleotides long aptamer named Broccoli. In contrast to Spinach derivatives, Broccoli possessed an efficient folding without tRNA scaffold and exogenous supply of Mg^{2+} . Also, Broccoli was found to be more thermostable (T_m 48°C) and not affected by flanking target sequences.

Another direction of the technology improvement was spectral turning of bluish-green chromophore **26** (excitation/emission at 447/501 nm) by chemical modifications.⁴⁷ Two useful red-shifted variants with almost unaltered affinity to Spinach2 were selected, namely green DFHBI-1T (**30**, excitation/emission at 482/505 nm) and yellowish DFHBI-2T (**31**, excitation/emission at 500/523 nm) (Figure 1).

Reversible binding of **26** to Spinach with a relatively low (micromolar) affinity results in unusual photobehavior of the complex. Under intense light illumination in microscopy, it bleaches very rapidly, but then recovers due to exchange with free chromophore molecules from the solution.^{48,49} This property can be used to achieve an extremely high photostability of the Spinach signal. For this, one should use light illumination at low repetition rate to give a sufficient time for the chromophore exchange and fluorescence recovery (Figure 3c).⁴⁸ Also, controllable photobleaching-recovery cycles can be used to discriminate between signals of Spinach-**26** and regular dyes or background fluorescence of the same color.⁴⁹

A number of practical applications of RNA aptamer-based fluorogenic complexes were suggested. First of all, it represents a genetically targeted fluorescent label for an RNA of interest.⁴² For example, it was shown that Spinach-**26** reporter can be readily used to follow gene expression in live bacteria at single cell level (Figure 3c) and to visualize intracellular distribution and dynamics of abundant transcription products of RNA polymerase III (e.g., 5S RNA) in living mammalian cells.^{42,50} At the same time, Spinach-based detection of RNA polymerase II-driven RNA species (mRNAs) is difficult to achieve,⁵¹ although one such an example was documented for Spinach2.⁴⁵ Spinach was also found to be useful for monitoring *in vitro* transcription.⁵²

Spinach can be used for construction of fluorescent sensors for various intracellular metabolites.⁵³ For this, a metabolite-binding RNA aptamer is inserted into Spinach structure in such a way that Spinach-**26** fluorescence strongly increases upon binding of the target metabolite.^{53,54,55} In a similar manner, Spinach can be fused with protein-binding aptamers to follow expression of endogenous target proteins in live bacterial cells.⁵⁶

To detect target RNAs, Bhadra and Ellington recently designed variants of Spinach that are triggered by hybridization with a sequence of interest.⁵⁷ Such “Spinach.ST” RNA folds into inactive conformation incapable to bind **26**. Upon hybridization with a specific sequence, Spinach.ST refolds into the fluorescent **26**-binding state. As of this moment, this technique has been tested *in vitro* only, but it has a great potential if successful in live cells.

Visualizing proteins. From HSA to Nuclear receptors

The heat map shown in Figure 2d demonstrated a number of selective successful Cro-protein interactions resulting in fluorescence turn-on. Led by the initial observation, we have synthesized compound **32a**, that shows unique sensitivity for human but not bovine serum albumin with FI = 72.⁵⁸ Another important group of proteins under study are nuclear receptors (NR), a superfamily of ligand-activated transcription factors that play crucial roles in a number of important biological and physiological processes.^{59,60} In recent years, the development of fluorescent probes has helped visualize the localization and trafficking of NRs inside cells. In particular, the use of the green fluorescent protein (GFP) fused to specific nuclear receptors has provided a powerful method for detecting both function and mobility of NRs *in vivo*.⁶¹ A disadvantage to fusion proteins, particularly to the ligand binding domain, is the potential interference with the interaction of the ligand binding domain with co-activator proteins essential for transcriptional regulation. More recently, studies have described various Human Estrogen Receptor alpha (ER α) ligand/fluorescent dye conjugates for the detection of ER α .^{62,63,64} However, the bulky conjugates of these ligands decrease the affinity of these ligands for the receptor.⁶⁵ Despite their visualization properties, these compounds are inadequate to serve as potential ligands for these receptors due to their activity profiles. Thus, the

use of potentially fluorescent small molecule *ligands* instead of the bulky GFP fusion protein or small molecule *conjugates* serves a two-fold advantage. Not only do these small molecules potentially serve as a new class of ligands for these receptors with activation profiles similar to other known ligands for these receptors, but the fluorescence capability of such molecules eliminates the bulkiness of a fusion protein that could distract the natural function of these proteins. In our preliminary study⁶⁶ we have found that several NRs (ER α , some of its variants, and few other NRs) can be activated using the synthetic GFP Cros. An initial search for the suitable ligand was based on the structural analogy between the non-fluorescent agonist and the Cro, confirmed by the docking modelling (Figure 4a). An activation of a NR by the groups of Cros homologs and isomers was performed in order to find the most active agonist. In some cases the activity of GFP Cros reached the one of the natural ligand (Figure 4b). An activation and localization of NRs in the presence of GFP Cros was performed in cells (Figure 4c) and in large microorganisms (Figure 4d).⁶⁷

Conclusions/perspectives

In this mini-review we demonstrated numerous applications of the new class of fluorogens, the synthetic FP chromophores. It is obvious that these molecules offer many advantages such as simple synthetic approach, broad spectral tunability, very low (if any) toxicity, and enormous FI when bound to the right host. In the end we outline few research directions that will enrich this area in the future. First, is a broader usage of “red” Cros. So far the majority of the demonstrated applications utilized the blue, green and yellow emission of the fluorophores. However, the rapidly expanding library of the red Cros¹⁶⁸ will open the door to the deep-tissue and low excitation-energy studies. The good alternative is, of course, a utilization of 2PA chromophores discussed earlier. The next unresolved issue involves a structural characterization of the Cros binding to various hosts. Up to date the only structure reported is the one of the Spinach-**26** complex.^{37,44} Indeed, an understanding of the structural binding motifs will lead to further optimization of these interactions, including better sensitivity and selectivity. Finally, the search for new proteins and other objects of interest is ongoing. The new exciting chromophore/host couples remain yet to be discovered!

Acknowledgements

The Moscow authors gratefully acknowledge support from the MCB program of the Russian Academy of Sciences, the Russian Foundation for Basic Research Grant grants 14-03-31162 mol_a and 13-04-01878a. M.S.B. acknowledges support by a stipend program of the President of

the Russian Federation. K.M.S. and L.M.T. acknowledge generous support from the National Science Foundation (CHE-1213047 and CHE-1425951). We are indebted to all collaborators listed in joint publications.

Figure 1. Structures of the synthetic FP chromophores discussed in the text. The subgroups of Cros are randomly color-coded and continuously numbered according to the corresponding

sections of manuscript. **1**: “native” GFP chromophore; **2 – 7**: AIE-active Cros; **8 - 13**: metal-sensitive GFP-based ligands; **14**: anion-sensitive Cro; **15 – 16**: Cros fused to macromolecules; **17 – 18**: GFP Cro-based polymers; **19 – 21**: locked Cros; **22 – 23**: Cros for binding to small hosts (octa-acids and cholates); **24 – 25**: Cros covalently attached to oligonucleotides for DNA-sensing; **26 – 31**: Cros for binding RNA aptamers; **32 – 34**: Cros for detecting nuclear receptors.

Figure 2. (a) Real-color photograph of **1** and **2a-c** crystals under daylight and UV-illumination. (b) Effects of metal complexation on **8a** fluorescence. Both figures (a) and (b) are reproduced with permission from Ref 1. Copyright 2012 American Chemical Society. (c) Schematic representation of the GFP Cro containing copolymer PEG-PNIPAM-Cro for bacterial detection. Adapted from Ref. 32 with permission from The Royal Society of Chemistry. (d) Heat map profiles of various analyte classes. Green bars correspond to fluorescence increase upon mixing. Adapted with permission from Ref. 36. Copyright 2013 American Chemical Society.

Figure 3. Structure of the RNA aptamer-chromophore complex. (a) Crystal structure of Spinach aptamer (stick representation) bound to the GFP-like synthetic chromophore **26** (spacefill representation, carbon atoms are shown green). (b) A closer look at the chromophore-binding region (stick representation). Images were created using PyMol (DeLano Scientific) from PDB 4TS2. (c) Time-lapse wide-field fluorescence microscopy of bacteria with Spinach/**26** under continuous (left) or low repetition rate (right) illumination. Note dramatic improvement of apparent photostability in the case of pulsed illumination scheme. Adapted with permission from Ref. 48. Copyright 2013 American Chemical Society. See the original reference for the experimental details.

Figure 4. GFP Chromophores as fluorescent agonists for nuclear receptors. (a) Modeling of GFP chromophore **33** in binding pocket of ERα with overlay of estradiol (pink). (b) Activation profile of chromophores in mammalian cells (HEK293T) expressing ERα. See Ref. 66 for more details. (c) Imaging of NIH 3T3 cells with the GFP Cros. Mammalian cells expressing ERα (Gal4DBD: ERαLBD) were incubated with **32b** (center) and **34** (left). As a negative control cells lacking ERα

were incubated with **32b** (right). (d) Visualization of estrogen-like NR in rotifer with compound **29**. Left – bright field image; center – confocal image; right – merged image.

References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- of special interest
- of outstanding interest

1 *Tolbert LM, Baldrige A, Kowalik J, Solntsev KM: **Collapse and recovery of Green Fluorescent Protein chromophore emission through topological effects**. *Acc Chem Res* 2012, **45**:171-181.

Recent review of the synthesis and utilization of the synthetic GFP chromophores

2 Baranov MS, Lukyanov KA, Yampolsky IV: **Synthesis of the chromophores of fluorescent proteins and their analogs**. *Russ J Bioorg Chem* 2013, **3**:223-244.

3 Follenius-Wund A, Bourotte M, Schmitt M, Iyice F, Lami H, Bourguignon J-J, Haiech J, Pigault C: **Fluorescent Derivatives of the GFP Chromophore Give a New Insight into the GFP Fluorescence Process**. *Biophys J* 2003, **85**:1839–1850.

4 Christensen MA, Jennum K, Abrahamsen PB, Della Pia EA, Lincke K, Broman SL, Nygaard DB, Bond AD, Brøndsted Nielsen M: **Highly fluorescent benzofuran derivatives of the GFP chromophore**. *RSC Adv* 2012, **2**:8243-8249.

5 Katritzky AR, Yoshioka-Tarvera M, El-Gendya BE-DM, Hall CD: **Synthesis and photochemistry of pH-sensitive GFP chromophore analogs**. *Tetrahedron Lett* 2011, **52**:2224–2227.

6 Petkova I, Dobrikov G, Banerji N, Duvanel G, Perez R, Dimitrov V, Nikolov P, Vauthey E: **Tuning the excited-state dynamics of GFP-inspired imidazolone derivatives**. *J Phys Chem A* 2010, **114**:10-20.

7 Xiang DS, Zhang CL, Chen L, Ji XH, He ZK: **Tricolour fluorescence detection of sequence-specific DNA with a new molecular beacon and a nucleic acid dye TOTO-3**. *Analyst* 2012, **137**:5898-5905.

-
- 8 Datta SG, Reynolds C, Goud YK, Datta B: **Interaction of YOYO-1 with guanine-rich DNA.** *J Biomol Struct Dyn* 2014, **32**:1155-1163.
- 9 Yan Q, Bruchez MP: **Advances in chemical labeling of proteins in living cells.** *Cell Tissue Res* 2015, **360**:179-194.
- 10 Mei J, Hong Y, Lam JWY, Qin A, Tang Y, Tang B-Z: **Aggregation-Induced Emission: The Whole Is More Brilliant than the Parts.** *Adv Mater* 2014, **26**:5429-5479.
- 11 **Ding D, Li K, Liu B, Tang B-Z: **Bioprobes Based on AIE Fluorogens.** *Acc Chem Res* 2013, **46**:2441-2453.
- 12 ** Liang J, Tang B-Z, Liu B: **Specific light-up bioprobes based on AIEgen conjugates.** *Chem Soc Rev* 2015, **44**:2798-2811.
- These two papers deal with rapidly developing area of AIE utilization for bioimaging.
- 13 Kwok RTK, Leung CWT, Lam JWY, Tang BZ: **Biosensing by luminogens with aggregation-induced emission characteristics.** *Chem Soc Rev* 2015, Advance Article DOI: 10.1039/C4CS00325J.
- 14 Fery-Forgues S, Veessler S, Fellows WB, Tolbert LM, Solntsev KM: **Microcrystals with enhanced emission prepared from hydrophobic analogues of the Green Fluorescent Protein chromophore via reprecipitation.** *Langmuir* 2013, **29**:14718-14727.
- 15 Huang GX, Zhang GX, Wu YS, Liao Q, Fu HB, Zhang, DQ: **Modification of the Green Fluorescent Protein chromophore with large aromatic moieties: Photophysical study and solid-state emission.** *Asian J Org Chem* 2012, **1**:352-358.
- 16 Shen X, Huang GX, Li K, Zhang GX, Zhang DQ: **Tuning the solid-state emission of the analogous GFP chromophore by varying alkyl chains in the imidazolinone ring.** *Sci Chin Chem* 2013, **56**:1197-1203.
- 17 *Tou S-L, Huang G-J, Chen P-C, Chang H-T, Tsaia J-Y, Yang J-S: **Aggregation-induced emission of GFP-like chromophores via exclusion of solvent-solute hydrogen bonding.** *Chem Commun* 2014, **50**:620-622.

Unusual mechanism of AIE

- 18 Hsu Y-H, Chen Y-A, Tseng H-W, Zhang Z, Shen J-Y, Chuang W-T, Lin T-C, Lee C-S, Hung W-Y, Hong B-C, Liu S-H, Chou P-T: **Locked ortho- and para-Core Chromophores of Green Fluorescent Protein; Dramatic Emission Enhancement via Structural Constraint.** *J Am Chem Soc* 2014, **136**:11805–11812.

19 Ikejiri M, Tsuchino M, Chihara Y, Yamaguchi T, Imanishi T, Obika S, Miyashita K: **Design and Concise Synthesis of a Novel Type of Green Fluorescent Protein Chromophore Analogue.** *Org Lett* 2012, **14**:4406–4409.

20 Huang GJ, Ho JH, Prabhakar C, Liu YH, Peng S-M, Yang J-S: **Site-selective hydrogen-bonding-induced fluorescence quenching of highly solvatofluorochromic GFP-like chromophores.** *Org Lett* 2012, **14**:5034-5037.

21 *Baldridge A, Solntsev KM, Song C, Tanioka T, Kowalik J, Tolbert LM: **Inhibition of twisting of a green fluorescent protein-like chromophore by metal complexation.** *Chem Commun* 2010, **46**:5686-5688.

First example of GFP Cros utilization for metal sensing

22 Fang XX, Wang Y, Wang D, Zhao GY, Zhang WW, Ren AM, Wang HY, Xu JW, Gao B-R, Yang W: **Synthesized blue fluorescent protein analogue with tunable colors from excited-state intramolecular proton transfer through an N–H...N hydrogen bond.** *J Phys Chem Lett* 2014, **5**:92-98.

23 Shi L, Li Y, Liu ZP, James TD, Long Y-T: **Simultaneous determination of Hg(II) and Zn(II) using a GFP inspired chromophore.** *Talanta* 2012, **100**:401-404.

24 Li Y, Shi L, Qin LX, Qu LL, Jing C, Lan MB, James TD, Long Y-T: **An OFF-ON fluorescent probe for Zn²⁺ based on a GFP-inspired imidazolone derivative attached to a 1,10-phenanthroline moiety.** *Chem Comm* 2011, **47**:4361-4363.

25 *Fang X, Li H, Zhao G, Fang X, Xu J, Yang W: **Blue fluorescent protein analogs as chemosensors for Zn²⁺.** *Biosens Bioelectron* 2013, **42**:308-313.

Extremely sensitive Zn²⁺ sensors.

26 Baranov MS, Lukyanov KA, Borissova AO, Shamir J, Kosenkov D, Slipchenko LV, Tolbert LM, Yampolsky IV, Solntsev KM: **Conformationally locked chromophores as models of excited-state proton transfer in fluorescent proteins.** *J Am Chem Soc* 2012, **134**:6025-6032.

27 Liu X-Y, Shi L, Ding Z, Long Y-T: **New insight into the application of GFP chromophore inspired derivatives: a F⁻ fluorescent chemodosimeter.** *RSC Adv* 2014, **4**:53557-53560.

28 Cacciarini M, Nielsen MB, de Castro EM, Marinescu L, Bols M: **beta-Cyclodextrin as a mimetic of the natural GFP-chromophore environment.** *Tetrahedron Lett* 2012, **53**:973-976.

-
- 29 Clark TB, Orr ME, Flynn DC, Goodson T: **Synthesis and optical properties of two-photon absorbing GFP-type probes.** *J Phys Chem C* 2011, **115**:7331-7338.
- 30 Clark TB, Ziolkowski M, Schatz GC, Goodson T: **Two-photon and time-resolved fluorescence spectroscopy as probes for structural determination in amyloid- β peptides and aggregates.** *J Phys Chem B* 2014, **118**:2351-2359.
- 31 Yuan L, Lin W, Chen H, Zhu S, He L: **A Unique Family of Rigid Analogues of the GFP Chromophore with Tunable Two-Photon Action Cross-Sections for Biological Imaging.** *Angew Chem Int Ed* 2013, **52**:10018-10022.
- 32 **Zheng Y, Li G, Deng H, Su Y, Liu J, Zhu X: **Temperature-induced fluorescence enhancement of GFP chromophore containing copolymers for detection of Bacillus thermophiles.** *Polym Chem* 2014, **5**:2521-2529.
- Very clever utilization of GFP Cro-based polymer.
- 33 Deng H, Zhu Q, Wang D, Tu C, Zhu B, Zhu X: **GFP-inspired fluorescent polymer.** *Polym Chem* 2012, **3**:1975-1977.
- 34 Baranov MS, Solntsev KM, Baleeva NS, Mishin AS, Lukyanov SA, Lukyanov KA, Yampolsky IV: **Red-shifted fluorescent aminated derivatives of conformationally locked GFP chromophore.** *Chem Eur J* 2014 **20**:13234-13241.
- 35 Frizler M, Yampolsky IV, Baranov MS, Stirnberg M, Gütschow M: **Chemical introduction of the green fluorescence: imaging of cysteine cathepsins by an irreversibly locked GFP fluorophore.** *Org Biomol Chem* 2013, **11**:5913-5921.
- 36 **Lee J-S, Baldrige A, Feng S, SiQiang Y, Kim YK, Tolbert LM, Chang Y-T: **Fluorescence response profiling for small molecule sensors utilizing the Green Fluorescent Protein chromophore and its derivatives.** *ACS Comb Sci* 2011, **13**:32-38.
- Combinatorial screening of GFP chromophores library.
- 37 *Vendrell M, Zhai D, Er JC, Chang Y-T: **Combinatorial Strategies in Fluorescent Probe Development.** *Chem Rev* 2012, **112**: 4391–4420.

Principles and applications of target - and diversity-oriented fluorescent libraries.

-
- 38 Baldridge A, Samanta SR, Jayaraj N, Ramamurthy V, Tolbert LM: **Activation of fluorescent protein chromophores by encapsulation. Steric and electronic effects in capsule-confined green fluorescent protein chromophores.** *J Am Chem Soc* 2011, **133**:712-715.
- 39 Baldridge A, Amador A, Tolbert LM: **Fluorescence turn on by cholate aggregates.** *Langmuir* 2011, **27**:3271-3274.
- 40 Riedl J, Menova P, Pohl R, Orsag P, Fojta M, Hocek M: **GFP-like Fluorophores as DNA Labels for Studying DNA-Protein Interactions.** *J Org Chem* 2012, **77**:8287-8293.
- 41 Wenge U, Wagenknecht H-A **Synthetic GFP Chromophore and Control of Excited-State Proton Transfer in DNA: An Alternative Concept for Fluorescent DNA Labels with Large Apparent Stokes' Shifts.** *Synthesis* 2011, **3**:502-508.
- 42 Paige JS, Wu KY, Jaffrey SR: **RNA mimics of green fluorescent protein.** *Science* 2011, **333**:642-646.
- 43 * Huang H, Suslov NB, Li NS, Shelke SA, Evans ME, Koldobskaya Y, Rice PA, Piccirilli JA: **A G-quadruplex-containing RNA activates fluorescence in a GFP-like fluorophore.** *Nat Chem Biol* 2014, **10**:686-691.
- 44 * Warner KD, Chen MC, Song W, Strack RL, Thorn A, Jaffrey SR, Ferré-D'Amaré AR: **Structural basis for activity of highly efficient RNA mimics of green fluorescent protein.** *Nat Struct Mol Biol* 2014, **21**:658-663.
- These two papers revealed unexpected structure of Spinach-DFHBI complex and provided basis for rational design of improved fluorogen-aptamer pairs.
- 45 Strack RL, Disney MD, Jaffrey SR: **A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat-containing RNA.** *Nat Methods* 2013, **10**:1219-1224.
- 46 ** Filonov GS, Moon JD, Svensen N, Jaffrey SR: **Broccoli: rapid selection of an RNA mimic of green fluorescent protein by fluorescence-based selection and directed evolution.** *J Am Chem Soc* 2014, **136**:16299-16308.

A new method of efficient fluorogenic aptamer selection was developed. An aptamer named Broccoli that significantly outperforms Spinach and Spinach2 was generated. Up to date, Broccoli appears to be the aptamer of choice for cell imaging.

47 * Song W, Strack RL, Svensen N, Jaffrey SR: **Plug-and-play fluorophores extend the spectral properties of Spinach.** *J Am Chem Soc* 2014, **136**:1198-1201.

Different positions of DFHBI chromophore were tested for substituents that do not affect binding to Spinach but change fluorescence spectra. A new chromophore DFHBI-1T with better spectral properties was selected.

48 Han KY, Leslie BJ, Fei J, Zhang J, Ha T: **Understanding the photophysics of the spinach-DFHBI RNA aptamer-fluorogen complex to improve live-cell RNA imaging.** *J Am Chem Soc* 2013, **135**:19033-19038.

49 Wang P, Querard J, Maurin S, Nath SS, Le Saux T, Gautier A, Jullien L: **Photochemical properties of Spinach and its use in selective imaging.** *Chem Sci* 2013, **4**:2865-2873.

50 Pothoulakis G, Ceroni F, Reeve B, Ellis T: **The spinach RNA aptamer as a characterization tool for synthetic biology.** *ACS Synth Biol* 2014, **3**:182-187.

51 Shin I, Ray J, Gupta V, Ilgu M, Beasley J, Bendickson L, Mehanovic S, Kraus GA, Nilsen-Hamilton M: **Live-cell imaging of Pol II promoter activity to monitor gene expression with RNA IMAGEtag reporters.** *Nucleic Acids Res* 2014, **42**:e90.

52 Höfer K, Langejürgen LV, Jäschke A: **Universal aptamer-based real-time monitoring of enzymatic RNA synthesis.** *J Am Chem Soc* 2013, **135**:13692-13694.

53 Paige JS, Nguyen-Duc T, Song W, Jaffrey SR: **Fluorescence imaging of cellular metabolites with RNA.** *Science* 2012, **335**:1194.

54 Kellenberger CA, Wilson SC, Sales-Lee J, Hammond MC: **RNA-based fluorescent biosensors for live cell imaging of second messengers cyclic di-GMP and cyclic AMP-GMP.** *J Am Chem Soc* 2013, **135**:4906-4909.

55 Strack RL, Song W, Jaffrey SR: **Using Spinach-based sensors for fluorescence imaging of intracellular metabolites and proteins in living bacteria.** *Nat Protoc* 2014, **9**:146-155.

56 Song, W., Strack, R.L., Jaffrey, S.R. **Imaging bacterial protein expression using genetically encoded RNA sensors.** *Nature Meth* 2013, **10**:873-875.

57 Bhadra S, Ellington AD: **A Spinach molecular beacon triggered by strand displacement.** *RNA* 2014, **20**:1183-1194.

58 Baldrige A, Feng S, Chang Y-T, Tolbert L M: **Recapture of GFP chromophore fluorescence in a protein host.** *ACS Comb Sci* 2011, **13**:214-217.

59 Novac N, Heinzel T: **Nuclear receptors: overview and classification.** *Curr Drug Targets Inflamm Allergy* 2004, **3**:335-346.

60 Gronemeyer H, Gustafsson JA, Laudet V: **Principles for modulation of the nuclear receptor superfamily.** *Nat Rev Drug Discov* 2004, **3**:950-964.

61 Htun H, Holth LT, Walker D, Davie JR, Hager GL: **Direct visualization of ligand occupied and unoccupied human estrogen receptor alpha reveals a role for ligand in the nuclear distribution of the receptor.** *Mol Biol Cell* 1999, **10**:471-486.

62 Adamczyk M, Reddy RE, Yu Z. **Synthesis of a novel fluorescent probe for estrogen receptor.** *Bioorg Med Chem Lett* 2002, **12**:1283-1285.

63 Asai D, Tokunaga T, Kondo K, Kawaguchi T, Takayanagi S, Shinmyozu T, Nakai M, Yakabe Y, Shimohigashi Y: **Direct measure of fluorescence intensity for efficient receptor-binding assay: Conjugates of ethinylcarboxyestradiol and 5(and 6)-carboxyfluorescein via α,ω -diaminoalkanes as a tracer for estrogen receptor.** *J Biochem* 2008, **143**:781-792.

-
- 64 Rickert EL, Oriana S, Hartman-Frey C, Long X, Webb TT, Nephew KP, Weatherman RV: **Synthesis and characterization of fluorescent 4-hydroxytamoxifen conjugates with unique antiestrogenic properties.** *Bioconjug Chem* 2010, **21**: 903-910.
- 65 Christoph S, Meyer-Almes F-J: **Novel fluorescence based receptor binding assay method for receptors lacking ligand conjugates with preserved affinity: Study on estrogen receptor α** *Biopolymers* 2003, **72**:256-263.
- 66 Duraj-Thatte A: **Fluorescent GFP chromophores as potential ligands for various nuclear receptors.** Ph.D. Thesis. Georgia Institute of Technology. 2012. Free access. URI: <https://smartech.gatech.edu/handle/1853/44764>.
- 67 Jones BL, Walker CL, Barry L, Azizi B, Tolbert LM, Williams LD, Snell TW: **Conservation of the estrogen receptor function in invertebrate reproduction.** In Preparation. 2015.
- 68 Fellows WB: **Combinatorial synthesis of new GFP- and RFP-like chromophores and their photophysical properties.** Ph.D. Thesis. Georgia Institute of Technology. 2014. Free access. URI: <https://smartech.gatech.edu/handle/1853/52318>.