DEVELOPMENT OF SPONTANEOUSLY BLINKING DNA-PAINT PLATFORMS FOR SUPERRESOLUTION MICROSCOPY IN LIVE CELLS

This project aimed at developing spontaneously blinking platforms on the grounds of point-accumulation for imaging nanoscale topography (PAINT) in order to enable superresolution imaging of intracellular proteins in live cells using stochastic approaches. We proposed a systematic study to investigate the possibility of implementing the DNA-PAINT technology to live cells, keeping features, such as low background, no-photobleaching, suitability for SMLM, general usability in mind. More particularly, we proposed three approaches: i) to design and synthesize low melting point peptide-nucleic acid (PNA) and DNA sequences carrying an intercalator dye ii) to combine FRET-PAINT and CLICK-PAINT principles into FRET-CLICK-PAINT and iii) to develop low affinity fluorogenic ligand activating protein tags.

I. Development of low melting point PNA-DNA sequences

Towards these aims we have synthesized a set of selected PNA strands (sequences were selected from *in silico* screens) and tested them with complementary ssDNA strands in order to identify potential hybrids suitable for temperature driven spontaneous blinking (i.e., melting points at around room temperature). Melting points and K_d values were determined by absorbance of the PNA-DNA hybrids at 260 nm. From these experiments we have concluded that the proposed temperature driven blinking system requires too short sequences to have melting points at around room temperature, which on the other hand sacrifices selectivity. Thus, this direction was abandoned.

In an alternative approach we have developed a double fluorogenic thiazole-orange (TO) derived probe (TO-tetrazine, TOT) that requires a bioorthogonal reaction with a protein of interest equipped site-specifically with a complementary bioorthogonal function (i.e., a cyclooctyne, BCN) on the one hand and interaction (intercalation) with DNA on the other in order to show highly intense fluorescence (Figure 1). We have demonstrated the suitability of this probe in sensing protein-DNA interaction in live cells. Figure 1 shows that fluorescence appears only in case of DNA interacting nuclear proteins (LaminA or H2B) equipped with a BCN motif via genetic fusion with a HaloTag self-labeling enzyme and treatment with Halo-BCN. In case of proteins (e.g., cytoskeletal TOMM20 or Vimentin) that do not interact with DNA no signal could be detected. These results indicate that the bioorthogonal reaction with BCN alone is not sufficient to trigger intense fluorescence and the presence of DNA in close proximity is also necessary. These results were published in *Chemosensors* **2022**, *10*, 37 (invited paper). Such double fluorogenic probes that can be targeted to specific recognition sites are promising candidates both in terms of signaling specific DNA-protein interactions and fine tuning the secondary interaction of the bioorthogonally anchored probe with DNA motifs to induce blinking.

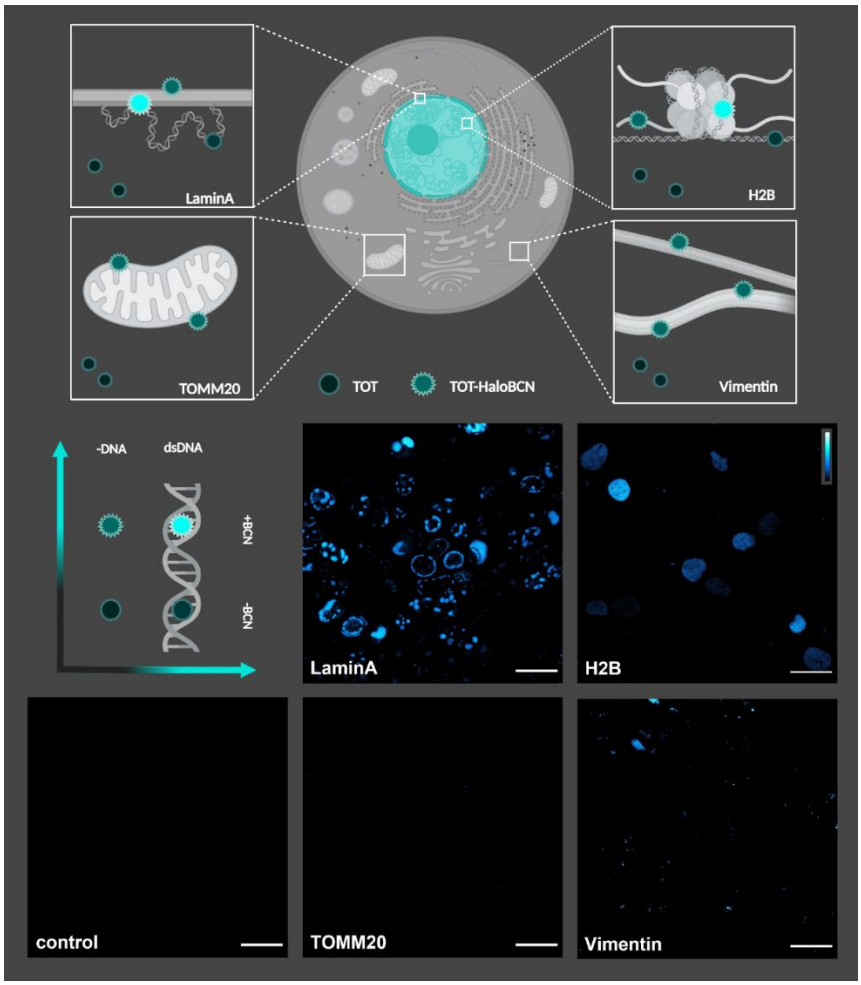

Figure 1. Schematic representation of labeling scheme with DNA–protein interaction sensing probe TOT (Upper panel). Changes in fluorescence intensity of the double fluorogenic probe TOT upon bioorthogonal conjugation (reactivity-based fluorogenicity) and interaction with DNA (structural fluorogenicity) is presented. Color calibration code shows the fluorescence intensity levels of TOT (middle panel, left). Confocal microscopy images of Lamin-HaloTag, H2B-HaloTag, TOMM20-HaloTag and Vimentin-HaloTag, expressing HEK293T cells treated with Halo-BCN (3 µM) and TOT (6 µM) (middle and lower panel). Control image refers to TOT-treated fluorescence of non-transfected cells. Spectral detection: λ_{exc}: 488 nm / λ_{em}: 500-800 nm; Scale bar = 25 µm.

II. Towards FRET-CLICK-PAINT systems

We have elaborated and developed methods for the modification of DNA strands in order to efficiently modify them e.g., with FRET probes or anchoring bioorthogonal motifs. We have successfully modified an antibody (suitable to specifically bind to mitochondrial protein TOMM20) with a Cy3-labeled 17-mer strand. In combination with a Cy5-labeled complementary strand we have successfully imaged the protein using the FRET signal of the probes. We have explored further oligonucleotide sequences that can be anchored to several target proteins (using our ca. 15-mer plasmid library suitable to express bioorthogonalized proteins), which have the potential (i.e., suitable K_d) to produce transient FRET signal (i.e., blinking) and image various proteins of interest with subdiffraction resolution. While this

approach could result in spontaneously blinking systems, the too complicated sample preparation and limited applicability concerned us therefore we sought for more generalizable approaches.

Part of this approach, however, we have developed novel probes with large Stokes shifts and/or improved fluorogenic character, and tested them in various superresolution microscopy setups even in multicolor labeling experiments. Unlike Cy probes, large Stokes shift probes have the potential to be involved in FRET systems with minimal or no cross-talk or bleed through. These results were reported in *Biomolecules* **2020**, *20*, 397 ; *Methods Appl. Fluoresc.* **2021**, *9*, 015006 and *Angew. Chem. Int. Ed.* **2022**, *61*, e202111855. We also explored the possibility of encoding a new ncAA enabling mutally orthogonal bioorthogonal labeling schemes (*Molecules* **2021**, *26(16)*, 4988) and developed a novel fluorogenic probe that enables mutually orthogonal bioorthogonal installation of probes onto suitable bioorthogonalized platforms (*Synthesis* **2022**, *54*, A–I).

III. Low-affinity fluorogenic ligand activating proteins

In line with our original objectives, we have started the elaboration of fusion protein tags capable of transiently accommodating fluorogenic ligands that become fluorescent upon binding. We hypothesized that labeling selected proteins fused to these Fluorogen Activating Protein (FAP) tags with appropriate fluorescent labels enabling energy transfer (FRET) between the two fluorophores will result in transient FRET and thus blinking in the acceptor channel. Such an approach would keep the beneficial non-bleaching features of DNA-PAINT and allows stochastic superresolution imaging without the need for complicated optical setups. Most importantly, this approach could lead to the extension of PAINT methods to live cells.

Accordingly, we obtained four different plasmids coding for FAP mutants with different spectral features and binding characteristics of specific fluorogenic ligands. The selected FAP variants were fused to model sequences coding for proteins belonging to distinct subcellular structures (plasma membrane, actin, tubulin, mitochondria) suitable for superresolution microscopy (SRM) study. We selected and synthetized the relevant FAP ligands from literature based on their spectral features and dissociation constants that could be relevant for FRET and SMLM purposes. We verified the correct functioning of these FAP fusion constructs and the ligands by fluorescent confocal microscopy, by combining different FAP mutants with suitable ligands in relevant concentrations (1-10 µM) based on dissociation constants.

Following the preliminary screening studies, we selected a FAP in combination with 4-hydroxy-3,5-dimethoxybenzylidene rhodamine (HBR3,5-DOM), which is known to be suitable for SMLM microscopy, although with handicap, as the authors found it challenging to find the labelled cells due to the small concentration of the fluorophore. Our approach, however, turned out to be suitable to solve this challenge, as we also introduced a second small fluorophore through GCE, without increasing the linkage error.

Next, we wished to modulate the photophysical properties of the FAP fluorescence by FRET. In order to create a platform to which various small-synthetic probes can be anchored to the FAP as FRET pairs of the FAP ligand and also to keep the linkage error minimal, we intended to incorporate a

bioorthogonal handle within the FAP tag through genetic code expansion (GCE) technology. Using this method, it is possible to incorporate bioorthogonalized non-canonical amino acids (ncAAs) such as bicyclo[6.1.0]non-4-yn-9-ylmethanol-lysine (BCNK) site-specifically at the rare TAG (Amber) stop codon. We have made a 6-membered mutant library including the ncAA encoding TAG mutation at various positions in the FAP tag, to identify the most efficient position for installing the fluorophores in terms of accessibility and FRET efficiency. For the sake of functional testing of the mutants, we fused them genetically to a membrane anchor peptide, Lyn11. Incorporation of the STOP codon was successful at all 6 sites. (Sites 35, 38, 104, 110, 117, 126). Of these, four positions allowed efficient labeling through tetrazine-cyclooctyne click recation. That is, membrane-specific labeling was possible with the test-dye, namely a probe developed in our laboratory earlier (Tet-HÁG35) in the case of Lyn11-FAP^{TAG} mutated at sites 35, 38, 104 and 117. When fluorogenic ligands were added, five mutants showed retained FAP-fluorescence: 35, 38, 104, 117 and 126, when 4-hydroxybenzylidene-rhodanine (HMBR) was added. Three of the double positive constructs are represented in Figure 2.

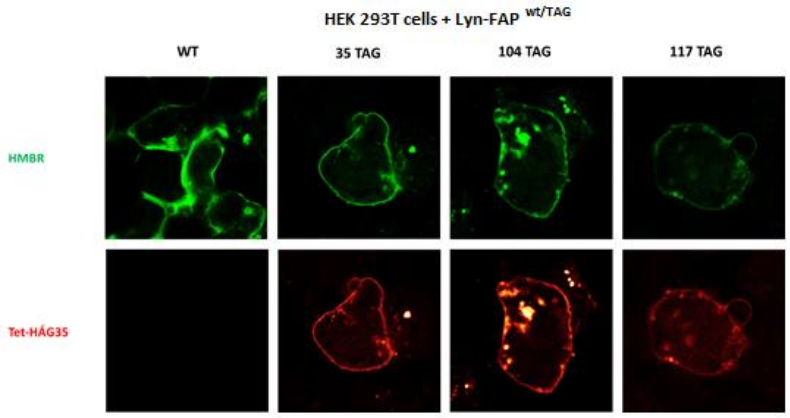

Figure 2. Detection of specific double labeling through a genetically incorporated biorthogonal handle (BCN-lysine) in the FAP tag. Mutants carrying an amber stop codon at the indicated site were in fusion with a plasma membrane anchor peptide (Lyn11). Upper row: HMBR ligand fluorescence. Lower row: Specific labelling through iEDDA reaction is verified by red fluorescence of Tet-HÁG35 in the three mutants shown.

We selected two mutants, FAP^{35TAG} and FAP^{104TAG} for further evaluation. We also used FAP fused to HaloTag, to test possible FRET. As a first step, we prepared a plasmid library using combinations of the FAPs and selected intracellular proteins. We screened several dyes (HÁG35, 4PheKum, SiR, Cy5, Janelia646 and SiR700) for FRET measurements using Fluorescence lifetime imaging (FLIM) (these experiments were carried out at the Department of Optics and Quantum Electronics, University of Szeged). Measurements were carried out so far on protein fusions with FAP^{104TAG} and FAP-HaloTag. We were able to detect the presence of appreciable FRET by FLIM measurements in the case of one dye pair on the FAP^{104TAG}-tag, namely the HBR-3,5DOM (HMBR was not suitable for SMLM) ligand combined with Tet-SiR700 probe (Figure 3). Weaker FRET was observed in the case of the FAP-HaloTag construct with the same fluorophore pair. This might be explained by the closer proximity of the two fluorophores within the FAP mutant. If needed, more efficient FRET might be achieved by involving further STOP codon mutants, to screen for the optimal constellation of FRET pairs where the relative dipole moments of the two fluorophores are positioned in a more favorable angle. We plan to test this option in the future.

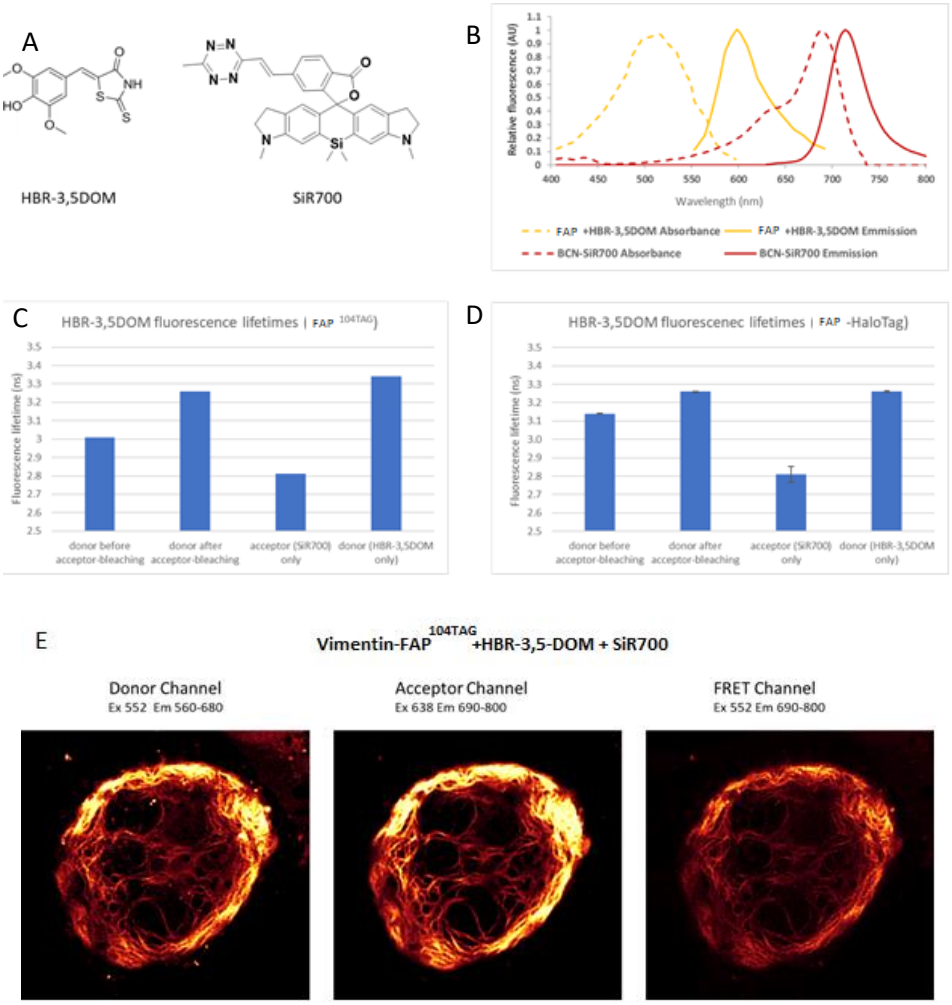

Figure 3. Features of, and measurements carried out on the fluorophore-pair HBR-3,5-DOM and SiR700 to detect FRET. A) Chemical structures of HBR-3,5-DOM and SiR700. B) Normalized absorbance and emission spectra of HBR-3,5-DOM and SiR700. C)-D) FLIM measurements on fixed cells, carried out using fluorescence lifetime imaging (FLIM) microscopy, and monitoring fluorescence lifetime increase of the donor after photobleaching of the acceptor. Double labeling of vimentin filaments in COS cells, with the FRET pair HBR-3,5-DOM and SiR700 was carried out with labelling of FAP104TAG with Tet-SiR700 or a FAP-Halotag fusion with Halo-BCN and Tet-SiR700 was used. Distance between the two fluorophores is somewhat larger in the latter case. E) Demonstration of double labeling of vimentin filaments in COS cells, with HBR-3,5-DOM and SiR700 using confocal microscopy. Emission in the FRET channel was also detected. Scale bar = 5 µm.

We also started assessing our double labeling system for SMLM microscopy. Our final aim would be to observe if improved image quality can be obtained via the FRET-enhanced labeling system by suppressing the background fluorescence of the donor (HBR-3,5-DOM) and acceptor (SiR700). In collaboration with the Advanced Optical Imaging Group (Department of Optics and Quantum Electronics, University of Szeged) we were able to generate an SMLM microscopic image of vimentin filaments double-labeled through FAP^{104TAG} with SiR700 in an upper plane of the cell (epifluorescent illumination) detected in the FRET channel (Figure 4.B.). However, we did not assess so far, if this setup yields an improved image than the donor alone.

The FAP-HaloTag construct was suitable for SMLM in the donor channel and transfected cells can be found easily. In this case, FRET efficiency was measured to be lower in the FLIM experiment. This was examplified by images taken of the microtubule network of COS cells in TIRF mode using the construct MAP4-FAP-HaloTag with HBR-3,5DOM and SiR700 fluorophores. Usability for FRET-SMLM has yet to be determined.

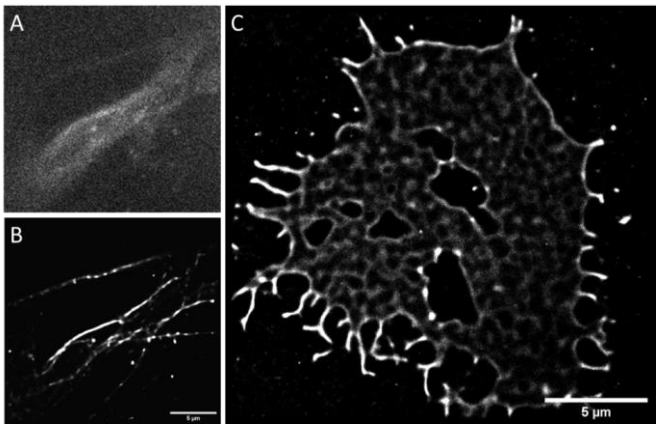

Figure 4. SMLM microscopic images obtained using different FAP variants. **A-B.** Double labeled vimentin filaments in COS cells, with FRET pair, HBR-3,5-DOM and SiR700 on FAP^{104TAG}. **A**. Conventional epifluorescent image illuminated at the acceptor excitation wavelength (exc. 647 nm, detection 690/70 nm). **B.** SMLM image of the same area illuminated at the donor excitation wavelength (488 nm) and detected at the acceptor wavelength (690/70 nm). Scalebar = 5 µm. **C.** Image demonstrating the applicability of the frFAP and HPAR-3OM pair for SMLM microscopy using a fusion construct of IL2Rα interleukin receptor subunit with frFAP, labelling the plasma membrane of COS cells (TIRF illumination) (exc. 647 nm, detection 690/70 nm). Scalebar = 5 µm.

Finally, a special mutant of FAP a far red applicable frFAP was also assessed for its capability for SMLM with the red-shifted 4-hydroxyphenylallylidene rhodanine analog HPAR-3OM, that emits in the NIR range. To our best knowledge, we were the first to observe its good applicability for SMLM microscopy, using a fusion construct of IL2Rα interleukin receptor subunit with frFAP, labelling the plasma membrane of COS cells (Fig. 4.C).

Conclusions

Our aims were to explore the possibilities of adapting the robust DNA-PAINT superresolution method to live cell imaging schemes. To this end we have explored three approaches one of which provided very promising results. This method employs the combination of low-affinity fluorogenic ligand activation proteins (FAP) with Förster-type of energy transfer. The promising results obtained within the third approach need to be further evaluated and confirmed. Furthermore, improvement of image quality and resolution should also be assessed. Moreover, live cell applicability of the above described setup should be demonstrated. We hope that this generalizable method enables the translation of the PAINT technique to live cell imaging. Furthermore, we also believe that it will enable to acquire images of intracellular structures with improved resolution. If further experiments confirm the viability of the FAP-FRET approach it provides a viable, generalizable method for live cell superresolution imaging of intracellular structures, which can be patented before publication. Experiments with frFAP should be completed before publication.

Dissemination of results

Publications

- Kormos, A.; Egyed, A.; Olvany, J. M.; Szatmári, Á.; Biró, A.; Csorba, Zs.; Kele, P.; Németh, K., **A bioorthogonal double fluorogenic probe to visualize protein–DNA interaction**, *Chemosensors* **2022**, *10*, 37. IF: 4.229, independent citations: 1.
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- Albitz, E.; Kern, D.; Kormos, A.; Bojtár, M.; Török, Gy.; Biró, A.; Szatmári, Á.; Németh, K.; Kele, P., **Bioorthogonal Ligation-Activated Fluorogenic FRET Dyads**, *Angew. Chem. Int. Ed.* **2022**, *61*, e202111855. IF: 16.823, independent citations: 2.
- Szatmári, Á.; Cserép, G. B.; Molnár, T. Á.; Söveges, B.; Biró, A.; Várady, G.; Szabó, E.; Németh, K.; Kele, P., **A Genetically Encoded Isonitrile Lysine for Orthogonal Bioorthogonal Labeling Schemes**, *Molecules* **2021**, *26(16)*, 4988. IF: 4.927, independent citations: 1.
- Török, Gy.; Cserép, G. B.; Telek, A.; Arany, D.; Váradi, M.; Homolya, L.; Kellermayer, M.; Kele, P.; Németh, K., **Large Stokes-shift bioorthogonal probes for STED, 2P-STED and multi-color STED nanoscopy**, *Methods Appl. Fluoresc.* **2021**, *9*, 015006. IF: 3.849, independent citations: 2.
- Németh, E.; Knorr, G.; Németh, K.; Kele, P., **A Bioorthogonally Applicable, Fluorogenic, Large Stokes-Shift Probe for Intracellular Super-Resolution Imaging of Proteins**, *Biomolecules* **2020**, *20*, 397. IF: 4.879, independent citations: 7.

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- Németh Evelin, Kern Dóra, Kormos Attila, Kele Péter – Nagy látszólagos Stokes-eltolódású fluorogén TBET rendszerek (szóbeli előadás, magyar). TTK Szerves Kémiai Intézet Szakmai Előadói Napok, Telki, 2020.01.23.
- Biró Adrienn, Németh Krisztina, Kele Péter, DNS plazmid-tár létrehozása nem-természetes aminosavak fehérjékbe építéséhez fluoreszcens mikroszkópiás és nanoszkópiás alkalmazásokhoz - Tavaszi Szél Konferencia Miskolc, 2021. 05. 28-29.
- Németh Evelin – Többszínű jelölésre alkalmas bioortogonális jelzővegyületek szintézise (szóbeli előadás, magyar). MTA Heterociklusos és Elemorganikus Kémiai Munkabizottság ülése, Patonay Tamás előadói nap, Budapest, 2021.09.03.
- Evelin Németh – Development of bioortogonally applicable fluorogenic probes for multicolor imaging (előadás, angol). Interdisciplinary Doctoral Conference, Pécs, 2021.11.12-13.
- Albitz Evelin - Többszínű jelölésre alkalmas bioortogonális jelzővegyületek szintézise (szóbeli előadás, magyar). MTA Bioorganikus Kémiai Munkabizottság ülése, Pécs, 2021.11.18.

- Szatmári Ágnes , Cserép B. Gergely, Molnár Tibor Á., Söveges Bianka, Biró Adrienn , Várady György, Szabó Edit, Németh Krisztina, Kele Péter, Kölcsönösen bioortogonális jelölésekre alkalmas, genetikailag kódolható nem-természetes aminosav fejlesztése, Az MTA Bioorganikus Kémiai Munkabizottság ülése, 2021. Október 21. Pécs
- Péter Kele, Bioorthogonally Activated Photoresponsive systems (Invited lecture), University of Rouen, France, 2022. 03. 01.
- Evelin Albitz - Development of bioortogonally applicable fluorogenic probes for multicolor imaging (előadás, angol). Second Symposium in Super-Resolution and Advanced Fluorescence Microscopy and István Ábrahám Memorial Workshop, Pécs, 2022.04.01-02.
- György Török, Gergely B. Cserép, András Telek, Dóra Arany, Zsófia László, Melinda Váradi, Éva Bakos, Csilla Özvegy-Laczka, László Homolya, Miklós Kellermayer, Péter Kele and Krisztina Németh. Large Stokes-shift bioorthogonal probes for STED, 2P-STED and multi-color STED nanoscopy. Second Symposium in Super-Resolution and Advanced Fluorescence Microscopy and István Ábrahám Memorial Workshop, Pécs, 2022.04.01-02.
- Albitz Evelin – Többszínű jelölésre alkalmas bioortogonális jelzővegyületek szintézise (szóbeli előadás, magyar). MTA Kémiai Biológia Munkabizottság ülése, Zeichmeister László előadói nap, Budapest, 2022.11.18.
- Egyed Alexandra – Xanténvázas fotolabilis vegyületek előállítása és vizsgálata. MTA Kémiai Biológia Munkabizottság ülése, Zeichmeister László előadói nap, Budapest, 2022.11.18.
- Kollár Virág, Kormos Attila, Kele Péter, Bioortogonálisan alkalmazható hatékonyan kioltott szilikorodolok szintézise, XV. Szent-Györgyi Albert Konferencia, Budapest, 2022. április 29-30.
- Náfrádi Dorka, Kern Dóra, Lövei Allegra, Kormos Attila, Kele Péter, In situ kinon-metid képzésre alkalmas fluorogén vegyületek szintézise, XV. Szent-Györgyi Albert Konferencia, Budapest, 2022. április 29-30.
- Attila Kormos, Alexandra Egyed, Jasmine M. Olvany, Ágnes Szatmári, Adrienn Biró, Zsóka Csorba, Péter Kele, Krisztina Németh, A Bioorthogonal Double Fluorogenic Probe to Visualize Protein–DNA Interaction, Peptide Chemistry and Chemical Biology Symposium, Balatonszemes, 30 May – 1 June 2022
- Dóra Kern, Attila Kormos, Allerga Lövei, Péter Kele, Bioorthogonally activatable fluorogenic self-labelling quinone methide probes, Peptide Chemistry and Chemical Biology Symposium, Balatonszemes, 30 May – 1 June 2022
- Péter Kele, Bioorthogonally Activated Photoresponsive systems (Invited lecture), Peptide Chemistry and Chemical Biology Symposium, Balatonszemes, 30 May – 1 June 2022
- Ágnes Szatmári, Gergely B. Cserép , Tibor Á. Molnár, Bianka Söveges, Adrienn Biró, György Várady, Edit Szabó, Krisztina Németh and Péter Kele, A genetically encoded isonitrile lysine for orthogonal bioorthogonal labeling schemes, Peptide Chemistry and Chemical Biology Symposium, Balatonszemes, 30 May – 1 June 2022
- Biró Adrienn, Unyi Zsombor, Cserép Balázs Gergely, Kele Péter, Németh Krisztina, Többszínű fluoreszcens jelölés kialakítása fehérjestruktúrák konfokális mikroszkópos és nanoszkópiás vizsgálatára. Fiatal Diplomások Fóruma 2022 - MTA Természetes Polimerek Munkabizottsága, az Óbudai Egyetem RKK Médiatechnológiai és Könnyűipari Intézete, PNYME Papíripari Szakosztálya és az Integrált Tudományok Szakkollégiuma összevont tudományos online ülése 2022. december 12.

Poster presentations:

- Evelin Albitz, Dóra Kern, Attila Kormos, Márton Bojtár, György Török, Adrienn Biró, Ágnes Szatmári, Krisztina Németh, Péter Kele – Bioorthogonal ligation-activated fluorogenic FRET dyads (poszter, angol). EMBO / EMBL Workshop: Chemical Biology, Heidelberg, Germany, 2022.09.05-08.
- Evelin Albitz, Dóra Kern, Attila Kormos, Márton Bojtár, György Török, Adrienn Biró, Ágnes Szatmári, Krisztina Németh, Péter Kele – Bioorthogonal ligation-activated fluorogenic FRET dyads (poszter, angol). Cell Bio An ASCB / EMBO Meeting, Washington DC, USA, 2022.12.03-07.
- Dóra Kern, Attila Kormos, Allegra Lövei, Péter Kele, Bioorthogonally activatable fluorogenic self-labelling quinone methide probes, Fluorescence Markers for Advanced Microscopy, École de Physique des Houches, France, 10-15 April, 2022
- Attila Kormos, Alexandra Egyed, Jasmine M. Olvany, Ágnes Szatmári, Adrienn Biró, Zsóka Csorba, Péter Kele, Krisztina Németh, A Bioorthogonal Double Fluorogenic Probe to Visualize Protein–DNA Interaction, Fluorescence Markers for Advanced Microscopy, École de Physique des Houches, France, 10-15 April, 2022
- Evelin Németh, Dóra Kern, Attila Kormos, Márton Bojtár, György Török, Adrienn Biró, Ágnes Szatmári, Krisztina Németh, Péter Kele – Bioorthogonally applicable fluorogenic energy transfer dyads for super-resolution microscopy (poszter, angol). EMBO / EMBL Symposium: Seeing is Believing – Imaging the Molecular Processes of Life, virtuális konferencia, 2021.10.05-08.