SYNSENSO

Cell-free synthetic biology for combinatorial biosensor design

Project no. 101072980

Deliverable D1.7
DNA-origami for kinase-regulator pair

Version 1.1

WP 1 – Building cell-free computing circuits

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Delivery date	28 August 2024
Dissemination level	Public
Type	Report

Funded by the European Union

Engineering and Physical Sciences Research Council

Funded by the European Union (GA 101072980) and supported by the UK Engineering and Physical Sciences Research Council. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the European Union or the European Research Executive Agency. Neither the European Union nor the granting authority can be held responsible for them.

Revision history

Date	Authors	Revision
16/08/2024	Maria Victoria Bussoletti Panizo	Draft version
20/08/2024	Maria Victoria Bussoletti Panizo, Karen Polizzi	First version
26/08/2024	Jörn-Frederik Wotzlaw	Review and editing
27/08/2024	Heinz Koeppl, Christiane Hübner	Internal review
28/08/2024	Heinz Koeppl	Final version

Contents

Abbreviations

Abbreviations	Details
POI	Protein Of Interest
POIs	Proteins Of Interest
eGFP	Enhanced Green Fluorescent Protein
CF	Cell-free
MP	Membrane protein
ssDNA	Single-stranded DNA
PCR	Polymerase Chain Reaction
PEG	Polyethylene glycol

Partner Short Names

Abbreviations	Details
TUDa	Technische Universität Darmstadt
TUE	Eindhoven University of Technology
IIT	Istituto Italiano di Tecnologia
TVU	Tor Vergata Universita degli Studi di Roma
DBS	Dynamic Biosensors GmbH
UBI	Ulisse Biomed S.p.A.
ICL	Imperial College London
ABV	Abvance Biotech S.L.
ACC	accelopment Schweiz AG
LEB	LenioBio GmbH
BRA	BRAIN Biotech AG
NUC	Nuclera Nucleics Ltd.
BME	Biomerieux SA
UNA	University of Naples Federico II

Executive Summary

This document reports on deliverable D.1.7 “DNA-origami for kinase-regulator pair”, activity carried out in collaboration with the SYNSENSO beneficiaries at Eindhoven University of Technology (TUE). The activities carried out during the 2-month secondment at TUE and future steps to apply it to the development of biosensors are reported.

Need for the Deliverable

This deliverable required a secondment to be fulfilled. The established secondment for this PhD project was carried out at TUE under the supervision of SYNSENSO beneficiary partner Prof. Ing. Tom de Greef and Dr. Indra Van Zundert, to gain essential expertise in DNA origami.

Objectives of the Deliverable

- Learn DNA origami nanotechnology principles
- Conjugate DNA origami with POIs

Outcomes

During the 2-month secondment, the principles of DNA origami were successfully acquired. A variety of DNA nanostructures were created, and different POIs were successfully conjugated to the DNA origami scaffold in a controlled and site-specific manner.

Next steps

Hands-on learning of this technique has provided valuable insights into the potential of DNA origami in cell-free systems. The next step will be to explore and develop suitable methods for integrating DNA nanotechnology into cell-free circuits.

1 Deliverable outcomes

1.1 Establishment of a DNA origami workflow

The primary objective of this deliverable, undertaken during a two-month secondment at TUE, was to acquire and establish proficiency in the DNA origami assembly technique for future implementing or applications in CF circuits.
DNA origamis are gaining considerable attention for their structural stability and precision. With DNA origami, it is possible to construct arbitrary shapes and position proteins with nanometre precision. Site-specific assembly of proteins can be achieved by direct conjugation of a protein to DNA. Alternatively, specific DNA nanostructures can be created to control the arrangement of proteins and lipid structures and to compartmentalise reactions in a defined space. DNA nanostructures have been used to study enzymatic cascades, where the distance and orientation of the enzymes play an important role in the reaction efficiency [1].
In the framework of this PhD project, DNA origami can be a valuable tool to compartmentalise enzymatic reactions, and to scaffold and bring closer the components to implement faster phosphorylation cascades in CF circuits.

DNA origami nanotechnology allows the precise construction of nanoscale structures that can be manipulated to create two-dimensional or three-dimensional shapes and patterns according to user specifications. The key components required for DNA origami assembly are:

1) Scaffold DNA: a long single-stranded DNA (ssDNA) molecule, typically derived from viral genomes.
2) Staple strands: short, complementary single-stranded DNA oligonucleotides designed to bind specific regions of the scaffold DNA.

When the staple strands are mixed with the scaffold DNA, they bind specific regions of the scaffold DNA through complementary base pairing. This binding process directs the scaffold to fold into the predetermined shape, resulting in the self-assembly of the desired DNA nanostructure.

For this deliverable, the scaffold ssDNA used was p7560, and staples of varying length from 20 to 45 base pairs were designed to create a final 18-helix bundle nanotube, hereby referred to as DNA nanorod. The scaffold DNA, staples design and final shape was based on *Cremers et al.* work [2]. The final dimension of the DNA nanorod was of 4670643,5 Da.

In addition to the DNA origami nanorod assembly, a site-specific protein conjugation to the DNA nanorod was also performed. This was achieved by incorporating modified staples that present a 25 to 30-base pair overhang that does not bind to the scaffold DNA; these modified staples are also called handles. Examples of different staples can be found in ***Table 1*****.** By controlling the positioning of the handles on the scaffold DNA, site specific protein assembly was performed. The POI, in this case eGFP, was designed to have a cystein residue at the N-terminus, to allow the conjugation of a oligo complementary to the handles present on the DNA nanorod, also called ‘anti-handles’. Red Atto647 dyes and N-ter Cys eGFP were conjugated to the DNA nanorod, as shown in ***Figure 1.***

Table 1: Examples of staples used for the assembly of the DNA nanorod. The "core oligo" staples have the whose sole function to bind to the ssDNA scaffold and shape it into its final nanorod form. The "modified handles" are staples containing the overhang necessary for conjugation of POIs. The "anti-handle" is the complementary sequence to the modified handle, attached to either the dye or eGFP for their conjugation to the DNA nanorod. Overhangs in the modified handles and their complementary sequences in the dyes and proteins are highlighted in bold and respectively coloured. Additional sequences can be found in the Appendix and in the reference work [2].

Name	Oligo
Core_oligo 1	AAGACACCGCCTAACTGGCGCGGTAAGCCAACAGAGAT
Core_oligo 2	GGAGAAAAATAACAGTACTTGAAACAAG
Modified_handle_Atto647	**ACTGACTGACTGACTGACTG**TTTGCTGCATTGTAAACGTTAATTAGAACC
Anti-handle on Atto647	**CAGTCAGTCAGTCAGTCAGT**-Atto647
Modified_handle_eGFP	**TCATACGACTCACTCTAGGG**TTTTTTTTCGCCAAATAATTCGCGTCTCTAAATC
Anti-handle on eGFP	**CCCTAGAGTGAGTCGTATGA**-NH2

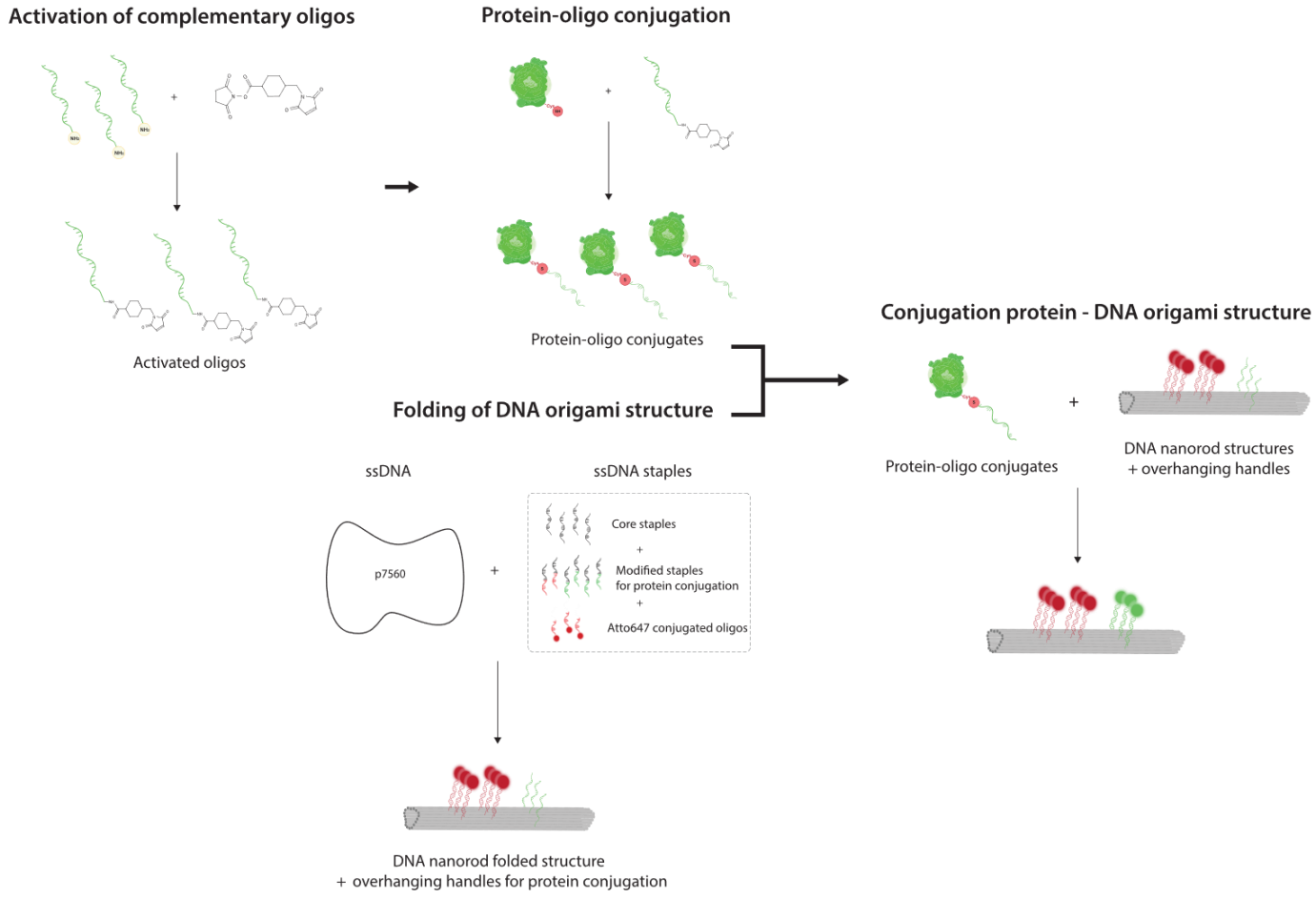

*Figure 1: DNA origami workflow. The first step undertaken was the **folding of the DNA origami structure** into a nanorod shape and the conjugation of a thermostable dye. The following step was to **activate the complementary oligos** (or anti-handles), and to **conjugate those with the POI**. The POI chosen was a recombinant eGFP presenting a N-ter cysteine residue through a chemical reaction. The protein-oligo conjugates were subsequently purified. The final step was the final **conjugation of the DNA nanorod structure with the oligo-conjugated eGFP**, resulting in a final DNA nanorod with eGFP conjugated in a site-specific manner.*

1.2 DNA nanorod folding designs

The core staples used in the assembly of the DNA nanorod were derived from *Cremers et al.* [2] work. To enable site-specific protein conjugation, some core staples were altered to include overhangs, transforming them into 'modified staples' for precise attachment of proteins or dyes at designated locations on the DNA nanorod.

After designing the staples, a tailored PCR with the ssDNA scaffold and an excess of staples was performed. The PCR cycles consisted in one denaturation cycle and two annealing cycles: the initial denaturation is to separate the viral scaffold and the staples, while the two annealing cycles' function is to slowly anneal the staples to the ssDNA scaffold. The temperature in the annealing cycles goes down of 1°C for each cycle to keep the forming DNA nanorod complex stable throughout the process.

After the PCR assembly reaction, a PEG precipitation was performed to remove the excess staples, and the formation of the nanorods was subsequently confirmed by gel imaging. The linearized ssDNA scaffold migrates more slowly on the gel compared to the assembled DNA nanorod, because of its more compact structure, resulting in a shift in molecular weight, as seen in ***Figure 2.***

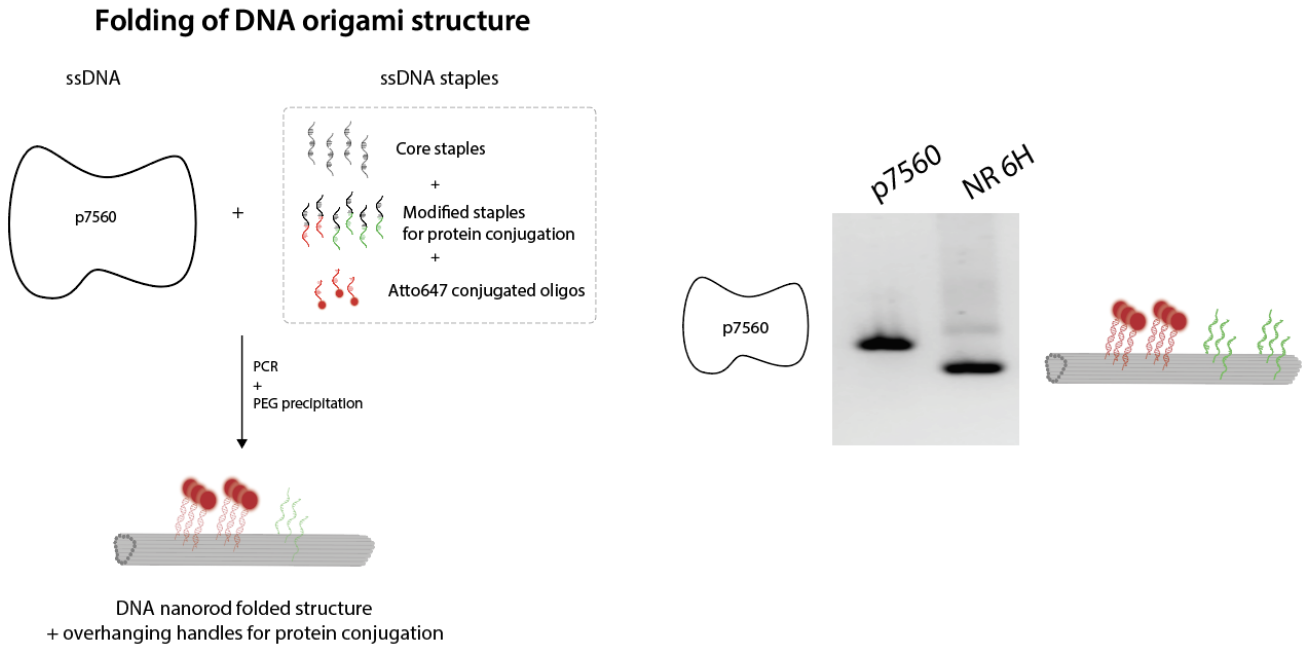

Figure 2: Left, schematic representation of the DNA nanorod folding procedure. On the right, verification of the DNA nanorod assembly in a 1.5% agarose gel in 0.5 TBE + Mg 10mM. This particular structure presents 12 handles: 6 were directly conjugated with the Atto 647 dye, and the remaining 6 are the free handles (hereby the name of NR 6H) for the subsequent conjugation of the eGFP.

Different modified staples from the previously designed pool were incorporated into the PCR assembly reaction, depending on the desired positioning of the protein within the DNA nanorod structure, as illustrated in ***Figure 3***. The differential use of these modified staples (in the ***Appendix***) enabled the creation of DNA nanorods with distinct sites for protein conjugation, allowing for various patterns of protein attachment. This differential protein conjugation among the DNA nanorod structures was subsequently confirmed following the conjugation process.

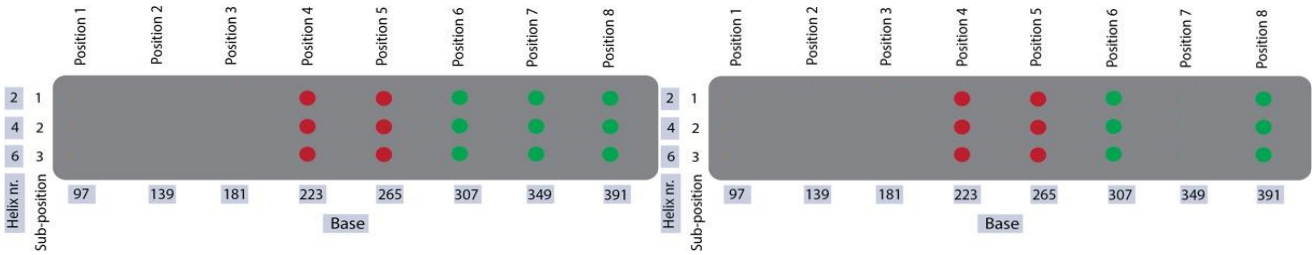

Figure 3: Schematic top view of the DNA nanorod, highlighting modified staples for the conjugation of the dye (in red), and eGFP (in green). The left panel shows a DNA nanorod with 6 conjugation sites for the dye and 9 for eGFP (NR 9H). For the assembly of this DNA nanorod, all eGFP modified staples were used. In the right panel, an alternative DNA nanorod with 6 binding sites for eGFP. The modified staples for position 7 were replaced with non-modified staples for the assembly of this DNA nanorod (NR 6H). See Appendix for modified staples sequences.

1.3 Protein conjugation with DNA complementary oligos (anti-handles)

The next step was to activate the DNA oligos and their conjugation to the POI. These oligos act as complementary anti-handles and allow the binding of the POI to the DNA nanorod. The activation method selected was the Sulfo-SMCC conjugation, which was particularly suited to the presence of an N-terminal cysteine residue in eGFP.
Sulfo-SMCC (sulfo-N-succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate) is a bifunctional crosslinker that facilitates the covalent attachment of proteins to other molecules. It contains two reactive groups: an N-hydroxysuccinimide (NHS) ester group and a maleimide group. The NHS ester group reacts with the amine present on the end of the modified staple, forming a stable amide bond. The maleimide group, on the other hand, specifically reacts with thiol groups, found in the terminal cysteine residue in the eGFP , as shown in the left panel of ***Figure 4.***

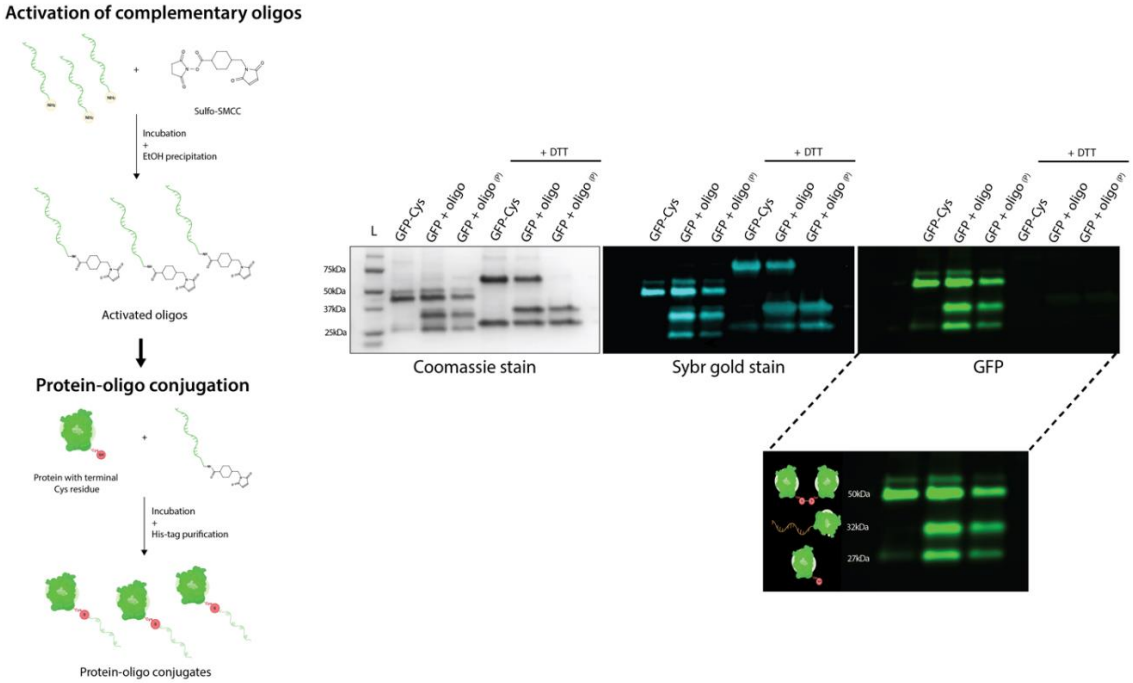

Figure 4: On the left, experimental scheme illustrating the Sulfo-SMCC activation of DNA anti-staples and their subsequent conjugation to eGFP. The Sulfo-SMCC reagent reacts with the terminal amines on the DNA oligonucleotides, activating them for conjugation with the N-terminal cysteine residue of eGFP. On the right, verification of protein-oligo conjugation is performed using gradient SDS-PAGE (10-25%). The gel is stained with various dyes to observe changes in the molecular weight of eGFP, which indicate successful conjugation or formation of side products. Prior to oligo conjugation, the eGFP with an N-terminal cysteine residue stored may appear as an undesired dimer. Post-Sulfo-SMCC activation, the gel reveals distinct bands corresponding to unconjugated eGFP (27 kDa) and the desired oligo-conjugated eGFP form (32 kDa). Legend: L = ladder, (P) = His-purified sample.

1.4 Site-specific conjugation of proteins to the DNA origami

After successfully assembling DNA nanorods with different staples, the oligo-conjugated proteins were attached to the DNA nanorod with a 1-hour long incubation at 37°C following a 2-hour long incubation at 22°C. After the incubation, the final complexes were purified either with size-exclusion chromatography or with an overnight dialysis. The accuracy of the site-specific protein was subsequently verified through gel imaging as shown in ***Figure 5***, confirming the correct assembly of the protein-DNA complexes.

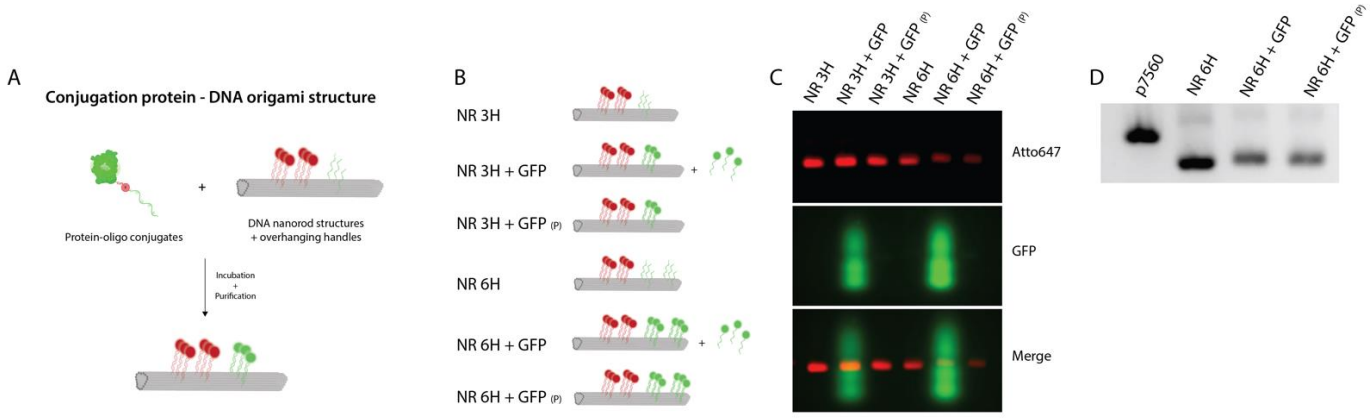

Figure 5: A) experimental workflow. The oligo-conjugated proteins were incubated with the pre-assembled DNA nanorods and purified. B) Representation of the different DNA nanorod structures analysed with gel electrophoresis. C) DNA origami structures visualised at different wavelengths on a 1.5% agarose gel in 0.5× TBE with 10 mM Mg. D) Comparison of the molecular weight of ssDNA scaffold with folded nanorods and protein-conjugated nanorods, determined by electrophoresis on a 1.5% agarose gel in 0.5× TBE with 10 mM Mg. Legend: (P) = PEG precipitation and purification.

2 Future plans

The next phase of this deliverable will explore several innovative applications of DNA origami nanotechnology.

One promising direction is the creation of compartmentalised spaces within cell-free systems using DNA origami structures. These compartments could provide a controlled environment for biochemical reactions, mimicking cellular processes at the nanoscale.

While the binding of histidine-kinase membrane proteins to DNA origami structures will also be investigated, it is worth recognising that the structure of membrane proteins and their requirement for a hydrophobic environment for proper folding may present a challenge to this approach.

Another possible research line would be the use of DNA origami to direct the assembly of lipids or nanodiscs, as previously reported in the literature [3–5]. By precisely positioning membrane mimics with DNA nanostructures, it may be possible to direct the formation of lipid bilayers or nanodiscs, providing new opportunities to study membrane-associated processes.

In addition, DNA origami will be used to achieve site-specific positioning of active soluble domains, allowing the study of potential interactions between these domains. This approach could provide valuable insights into the spatial dynamics of kinase-regulatory pair reactions in CF circuits.

3 References

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4 Appendix

4.1 Modified staples used for dye conjugation

Handle sequence (overhang)	**ACTGACTGACTGACTGACTG**
Anti-handle on the dye	CAGTCAGTCAGTCAGTCAGT-Atto647

mC4.1	**ACTGACTGACTGACTGACTG**TTTGCTGCATTGTAAACGTTAATTAGAACC
mC4.2	**ACTGACTGACTGACTGACTG**TTCTCATATATAAGAGGTCATTTAGTTTTG
mC4.3	**ACTGACTGACTGACTGACTG**TTCCAGAGGAAGAGTATTTTTCATGAGGAATCCACAG
mC5.1	**ACTGACTGACTGACTGACTG**TTGCGGGCCGTTAAATCAGCTCATTGCGGG
mC5.2	**ACTGACTGACTGACTGACTG**TTAGAAGCCAATATAATGCTGTAACGACGA
mC5.3	**ACTGACTGACTGACTGACTG**TTTAAAAACAACGTAAACGAGGGTAGCAACTGTCGTC

4.2 Modified staples used for eGFP conjugation

Handle sequence (overhang)	**TCATACGACTCACTCTAGGG**
Anti-handle on eGFP	CCCTAGAGTGAGTCGTATGA-NH2

mL6.1	**TCATACGACTCACTCTAGGG**TTTTTTTTCGCCAAATAATTCGCGTCTCTAAATC
mL6.2	**TCATACGACTCACTCTAGGG**TTTTTTGGTTGTAAGTACGGTGTCTGGAGGCATA
mL6.3	**TCATACGACTCACTCTAGGG**TTTTTTGTAAGAGACGAGAATTTGCGGGATCGTCATTTTGC
mL7.1	**TCATACGACTCACTCTAGGG**TTTTTTTTCCGGCATTAAATGTGAGCGAAGAATT
mL7.2	**TCATACGACTCACTCTAGGG**TTTTTTAGCAAAACCAATTCTGCGAACACATTCA
mL7.3	**TCATACGACTCACTCTAGGG**TTTTTTACTAATGTTTAATTATATATTCGGTCGCAGAAAGG
mL8.1	**TCATACGACTCACTCTAGGG**TTTTTTGACGACAAACAAACGGCGGATTAGTAGT
mL8.2	**TCATACGACTCACTCTAGGG**TTTTTTAGCATTAATTTCGCAAATGGTATTACAG
mL8.3	**TCATACGACTCACTCTAGGG**TTTTTTGTAGAAATTAAGAAGTTGCGCCGACAATCGTTGAA