# RELAZIONE SCIENTIFICA FINALE

SHORT TERM MOBILITY PROGRAM 2017

**Beneficiary**: PERUGINO Giuseppe

**Institute**: Institute of Bioscience and BioResources

**Position**: Staff Researcher (level III)

**Program Title**

A thermostable *protein-tag* for DNA-protein 3D nanostructures

## INTRODUCTION

Over the past three decades, DNA, the genetic information carrier in most living organisms, has seen an ever-expanding role as a material for the construction of nanoscale objects [Wang *et al.*, 2017]. One technique in particular, known as *DNA Origami*, has opened up the ability for researchers to design arbitrarily shaped complex three-dimensional (3D) nanostructures (Figure 1). “Origami” refers to the art of folding and sculpting a flat sheet of paper into arbitrarily shaped objects. *DNA Origami* is a process of molecular self-assembly: a long single-stranded DNA (scaffold), typically M13 phage genomic DNA (ca. 7,000 bp), is folded into prescribed objects by hundreds of short synthetic DNA oligonucleotides, typically 20-60 bp long, which are designed to be complementary to different parts of the scaffold DNA [Wang *et al.*, 2017] (Figure 1A).

In biology, the spatial and temporal arrangement of biomolecules into well-defined complexes leads to enhanced activity or new functionality. The ability to mimic these complexes through nanofabrication with *DNA Origami* is a useful tool for better understanding native multi-protein systems in an effort to construct more efficient enzymatic nanoreactors, conduct single-molecule biophysical studies, or achieve super-resolution optical imaging [Wang *et al.*, 2017]. Advances in the ability to functionalize DNA nanostructures with proteins have enabled the synthesis of a wide variety of nanofabricated biomolecule systems [Saccà and Niemeyer, 2012]. Although simpler DNA nanostructures had been previously used for the assembly of multi-enzyme systems, Yan and colleagues were the first to use *DNA Origami* to scaffold a more complex, three-protein system [Fu *et al.*, 2012]. Using a 2D rectangular *DNA Origami* tile, the authors were able to investigate the effects of protein distance on a bi-enzymatic process using glucose

oxidase and horseradish peroxidase. The authors found that placing a non-enzymatic ‘‘bridge’’ protein between the two enzymes could increase the reaction rate even further (Figure 2A).

3D *DNA Origami* nanostructures have also been used for modulating and studying enzymatic behavior, such as using a ‘‘nanocage’’ composed by two half-shell structures, to encapsulate multiple enzymes improving their reaction efficiency [Zhao *et al.*, 2016] (Figure 2B).

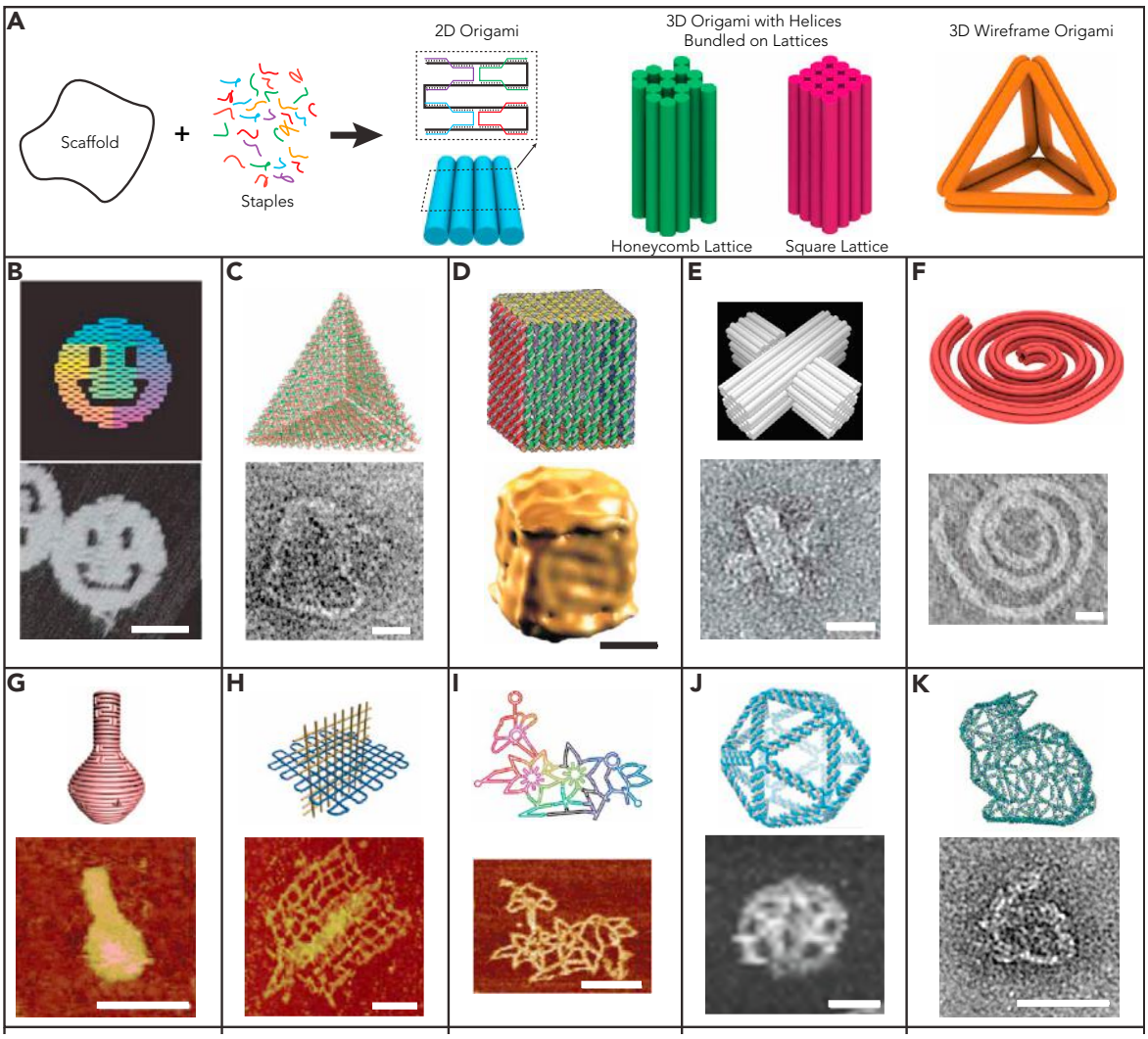


**Figure 1.** *Overview of DNA Origami design strategies and structures*. (A) *DNA Origami* design strategies for constructing 2D and 3D objects. (B) *DNA Origami* smiley face; (C and D) a hollow tetrahedron (C) and cube (D) formed from the folding of flat *DNA Origami* sheets; (E) a slotted cross constructed from honeycomb DNA lattice; (F) a curved 6-helix bundle spiral-like object; (G) a nanoflask built from concentric rings of DNA helixes; (H) a 3D gridiron structure based on DNA four-arm junctions; (I) a wireframe flower-and-bird pattern; (J) a wireframe icosahedron; (K) a wireframe rabbit (from [Wang *et al.*, 2017]).

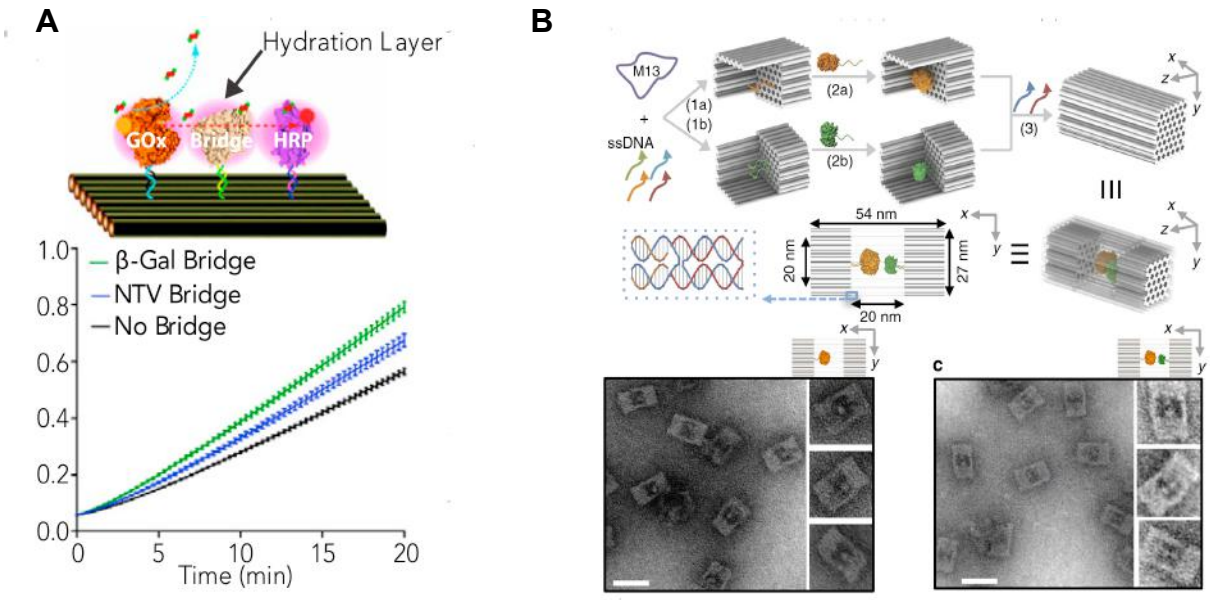


**Figure 2.** *DNA Origami for Nanofabrication with Biomolecules*. (A) Assembly of a GOx-HRP cascade on *DNA Origami* with a protein bridge in between to facilitate $H_2O_2$ diffusion through a connected hydration layer and increased reaction rates (from [Fu *et al.*, 2012]); (B) design and characterization of DNA nanocage-encapsulated enzymes with controlled stoichiometry (from [Zhao *et al.*, 2016]).

*DNA Origami* combined with proteins and enzymes are crucial for drug delivery and the development of nano-biosensors. One of the most difficult challenges is the encapsulation of enzymes in closed and hollow DNA-based structures: these “nano-boxes” could be useful for triggering enzyme activities in the presence of signals and / or cellular stimuli. However, the thermal assembly process of these 3D DNA structures starts at high temperatures, which greatly affects the stability of proteins, thus limiting this innovative technology. Moreover, as demonstrated with other approaches, the immobilization of biocatalysts could affect their activity, because of the steric hindrance of the support (in this case the DNA structure) and the wrong orientations of the enzyme toward its substrate [Zhao *et al.*, 2016].

The use of thermostable *protein-tags* fused to equally thermostable enzymes could improve and, in some cases, make possible the production of these *DNA Origami*-protein complexes, taking advantage of the direct immobilization of the *protein-tag* moiety on the DNA support, and leaving thus the enzymes free to proceed in their catalysis.

**AIMS**

*We aim to demonstrate the utility of a thermostable protein-tag (SsOGT-H$^5$) for a spatially-specific decoration of DNA scaffolds, in particular on closed and hollow 3D structures. The immobilization of SsOGT-H$^5$ will be performed on the already formed nano-structures, as well as by introducing this tag during their assembly at high temperatures, increasing the possibility that the protein enters in the cages.*

## LAB ACTIVITIES

All the experiments described below were performed at the Center for Medical Biotechnology (ZMB) of the University of Duisburg-Essen (Universitätstraβe 4, 45117 Essen, GERMANY), under the supervision and the collaboration of Dr. Barbara Saccà and her PhD student (Andreas Jaekel).

*Ss*OGT-H$^5$ (hereafter in H$^5$) is a variant of the *O*$^6$-alkylguanine-DNA-alkyl-transferase (AGT, OGT or MGMT) from the thermophilic microorganism *Sulfolobus solfataricus*. [Perugino *et al.*, 2012, 2015; Vettone *et al.*, 2016; Morrone *et al.*, 2017]. OGTs are ubiquitous proteins involved in the direct repair of alkylation damage in DNA by a one-step mechanism, catalyzing the transfer of the alkylic group from *O*$^6$-alkyl-guanine (or *O*$^4$-alkyl-thymine) to a cysteine residue in their own active site [Daniels *et al.*, 2004; Fang *et al.*, 2005; Yang *et al.*, 2009; Pegg, 2011]. AGTs are called “suicide” or “kamikaze” proteins because the stoichiometry of the reaction is 1:1 and the alkylated form of the protein is irreversibly inactivated [Yang *et al.*, 2009; Fang *et al.*, 2005] (Figure 3A). The covalent nature of the alkylated form of AGTs and their small protein size (< 20.0 kDa) opened new biotechnological approaches, making them interesting tools for the utilization as *protein tags* in *in vivo* and *in vitro* specific labelling of proteins [Keppler *et al.*, 2003, 2004; Gautier *et al.*, 2008]. Commercial variants of the human AGT (SNAP®- and CLIP®-*tag*, New England Biolabs) can be covalently labelled with a large number of chemical groups conjugated with the *O*$^6$-benzyl-guanine (BG), a classical AGTs inhibitor (Figure 3B).

In a previous work, Dr. Saccà already decorated the surface of a 2D DNA scaffold by using two orthogonal substrate specificity activities, the SNAP-*tag*® and another *protein-tag*, the *Halotag*® (Promega) fused at proteins of interest [Saccà *et al.*, 2012].

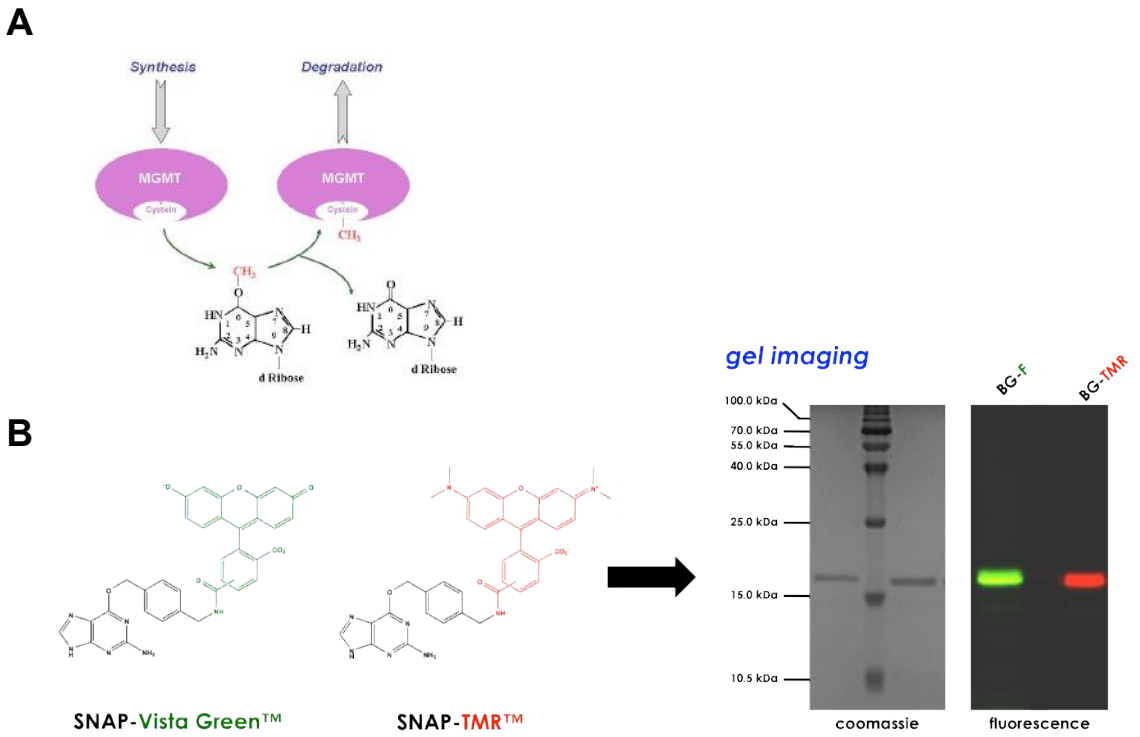


**Figure 3:** *The utilization of AGTs as protein-tag.* (A) Reaction mechanism of AGTs in the presence of their natural methylated DNA substrate; (B) AGT specific labelling with commercially available fluorescent probes (in green and red) conjugated with the inhibitor $O^6$-benzyl-guanine (in black).

Our attempt will be the utilization of our "thermostable SNAP-*tag*" (the above described $H^5$ variant from *S.solfataricus*) for the decoration of a DNA scaffold having a peculiar hollow structure: in the lab of Dr. Saccà, by using the M13 DNA single strand scaffold and 232 oligonucleotides (*staple oligos*), a parallelepiped with a hexagonal base DNA structure was prepared, in its closed and semi-closed form (Figure 4A). These structures possess in their cavity one-to-six oligonucleotides (*protruding arms*), which are complementary to oligonucleotides conjugated with $O^6$-benzyl-guanine, the substrate of the $H^5$ protein (Figure 4B). The activity of the enzyme leads to the covalent bond with the oligonucleotide and, subsequently, the association of the protein inside the cage by a double strand formed DNA (Figure 4C).

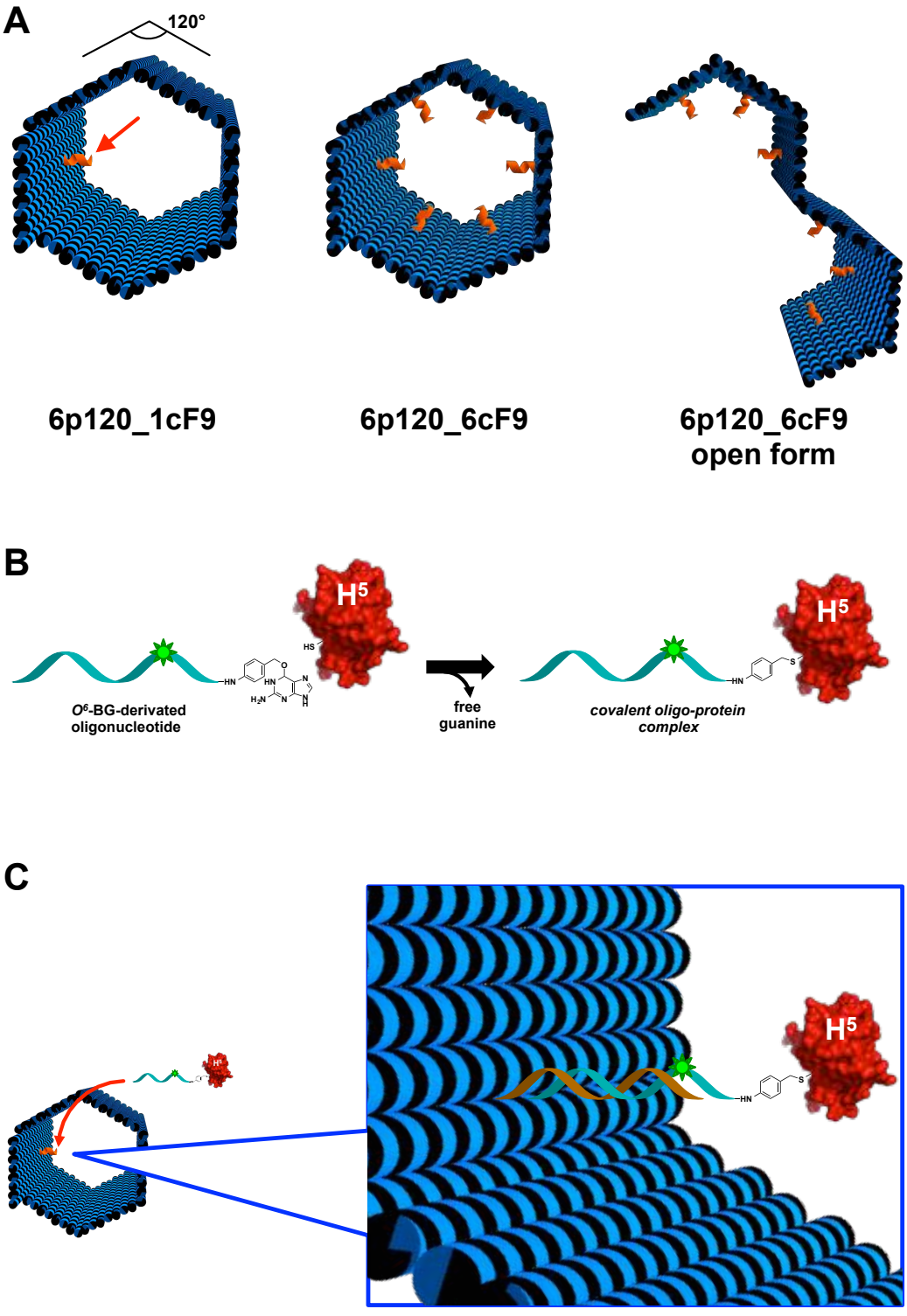


**Figure 4:** *The production of the DNA Origami-H*$^5$ *conjugate.* (A) The structure is composed by six 2D-sides of DNA (6p, each cylinder schematically represents a DNA double strand) linked by 120°-angle hinges. Inside the cage, it is possible to insert one or six *protruding arms* (cF9, *in orange*). (B) *Ss*OGT-H$^5$ irreversibly reacts with an *O*$^6$-BG conjugated with a fluorescent oligonucleotide (BG-F9(16)-FAM, *green star*), whose sequence is complementary to the *protruding arm* in the cage. (C) The annealing between cF9 and the H$^5$-FAM-oligo allows inserting the protein in the DNA structure.

The first experiment was the testing of the activity of $H^5$ against the BG-F9(16)-FAM for the production of the protein-oligo covalent complex (Figure 4B), as in the following procedure:

- .. 1.5 nmol of BG-oligo;
- .. 7.5 nmol of H5 (ca 150 μg);
- .. $V_{tot}$ 100 μL in Fluo Reaction Buffer 1X (FRB, phosphate buffer 50 mM; NaCl 150 mM; DTT 1 mM; pH 7.3);
- .. incubation for 60 min at 50 °C;
- .. added 40 μL of BG-agarose bed resin in $V_{tot}$ 200 μL of FRB 1X;
- .. incubation for 16 h at 4 °C in 2 mL reaction tube in roll shaker;
- .. centrifugation at 13000 g for 5 min;
- .. discard the resin and store the surnatant.

Because of the excess of the protein (1:5 oligo:protein ratio), we used the BG-agarose resin to remove the unreacted $H^5$ by covalent trapping in a one step purification procedure (Figure 5A).

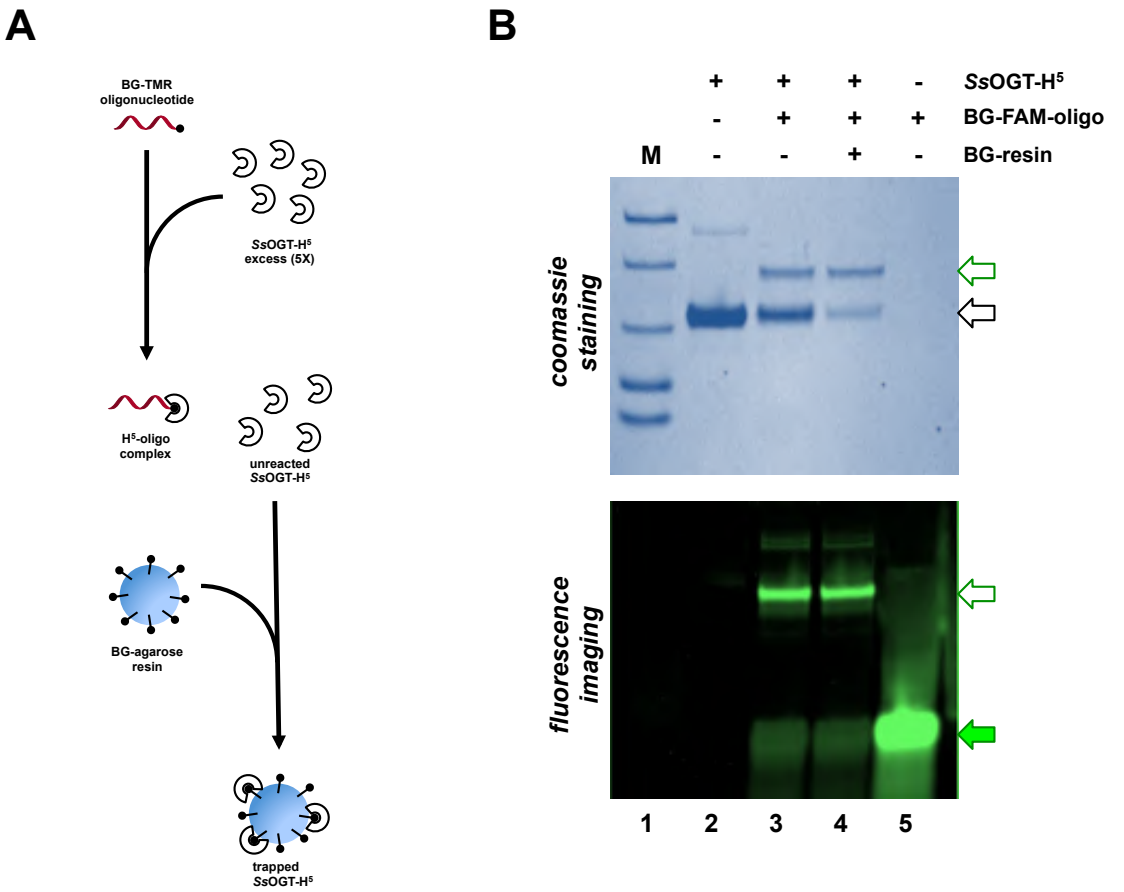


**Figure 5:** *Production of the $H^5$-oligo covalent complex.* (A) Scheme of the protocol used. The BG-agarose resin allows the purification of the complex by entrapping the unreacted $H^5$. (B) SDS-PAGE and gel imaging of the experiment described in the text. *Black open*, *green open* and *green closed arrow* stand for the $H^5$ protein, the $H^5$-oligo complex and the free oligo, respectively. M, molecular weight marker.

As shown in Figure 5B, $H^5$ reacted almost completely with the BG-F9(16)-FAM oligo (*lane 3*), as well as the resin almost completely entrapped the unreacted $H^5$ protein (*lane 4*). These results gave important information about reaction conditions for the covalent bond between the protein and the oligonucleotide, to be applicable in the subsequent experiments to insert that complex in the *DNA Origami* cage.

At the same time, the efficiency of the annealing of the BG-F9(16)-FAM oligo on the *DNA Origami* structures was tested: M13 single strand DNA was mixed with 10X *staple oligos* and 20X BG-F9(16)-FAM, and incubated 10 min at 65 °C, cooling 2 min/°C to 25 °C, kept at 10 °C after assembly program was finished (Figure 6).

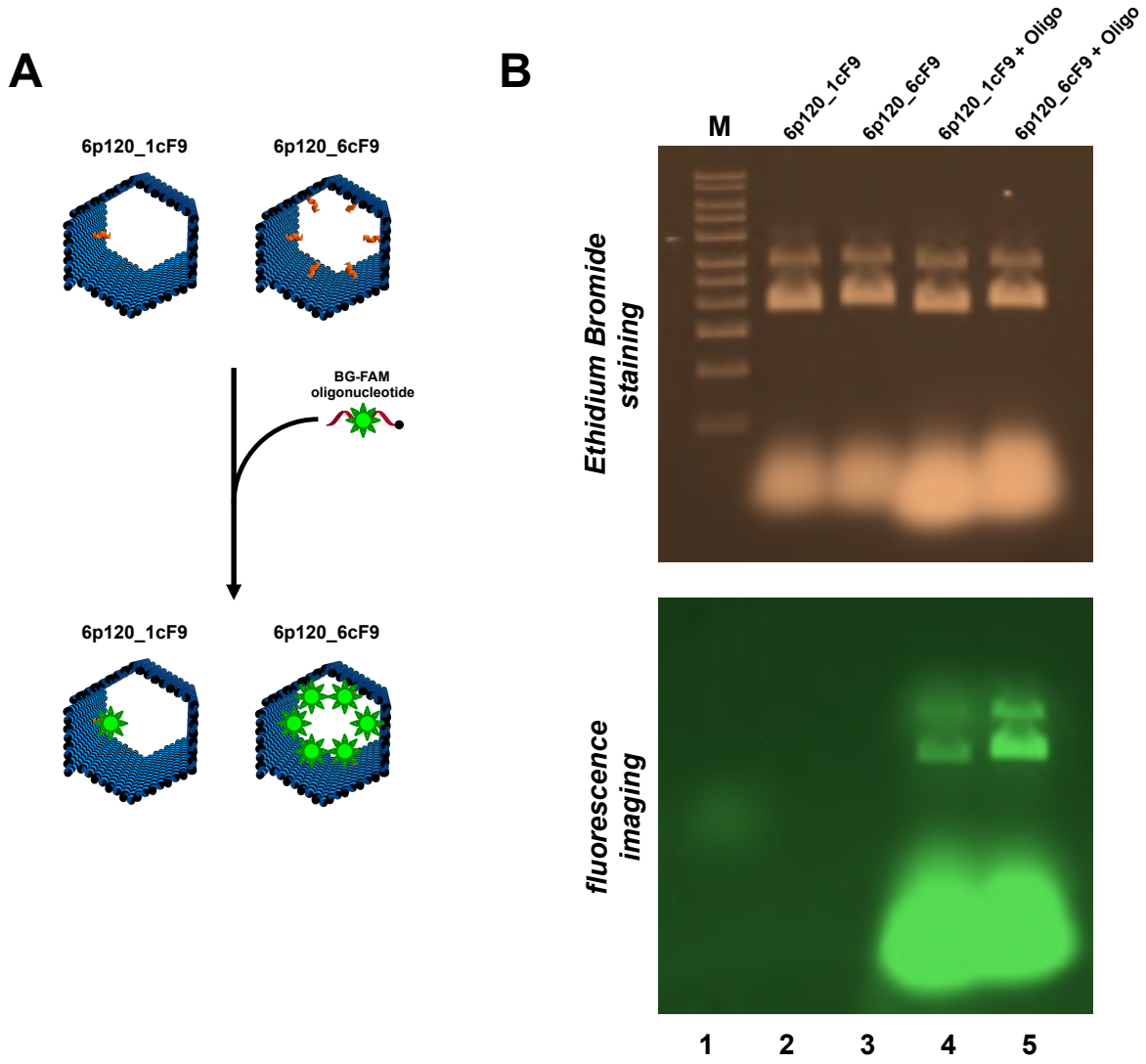


**Figure 6:** *Production of the DNA Origami cage containing fluorescent BG-F9(16)-FAM oligonucleotide.* (A) Scheme of the protocol used. (B) Running of *DNA Origami* samples by agarose gel and imaging by Ethidium Bromide staining and FAM filter. M, 1.0 kbp molecular weight marker.

As expected, despite the visualization by ethidium bromide staining, the FAM fluorescent intensity is directly correlated to the amount of oligo annealed with *the protruding arm*, giving a stronger signal in the 6p120_6cF9 structure (*lane 5*).

As control, we first analyzed the encapsulation of the $H^5$-oligo conjugate in the already formed DNA cage (as shown in Figure 4C): 10 nM of this structure was mixed with 20X of the conjugate for 60 min at 40 °C, then the mixture was 100 kDa MWCO-filtered, in order to remove the excess of the conjugate. Finally, the sample was analyzed by agarose gel and by Atomic Force Microscopy (AFM).

Agarose gel analysis confirmed that the $H^5$-oligo conjugate interacted with the cage by the annealing of the BG-F9(16)-FAM oligo with its complementary *protruding arm* oligo in the DNA structure (Figure 7A). As expected, this was better noted for the cage containing six *protruding arms* (6p120_6cF9), which was slower in the gel running and more intense in the FAM fluorescent analysis (*lanes 4 and 6*). The shift of the DNA band on the gel due to the presence of the $H^5$-oligo conjugate was also confirmed by the AFM: although the $H^5$ protein is very small (ca. 20 kDa, in comparison with the MDa dimension of the DNA cage), it was possible to visualize some spots (*yellow arrows* in Figure 7B) on structures.

Finally, we proceed with the *one-step encapsulation* of the conjugate in the cage: 10 nM of the DNA was mixed with 20X of the $H^5$-oligo conjugate or with the separated $H^5$ (20X) and the BG-F9(16)-FAM oligo (10X) after the initial 10 of the 20 min at 65 °C step of the assembly program.

The encapsulation of $H^5$ in the 6p120_1cF9 structure was very difficult to determine in both the above-mentioned analyses. Although the shift is not clearly visible, mainly for the reaction where the $H^5$ and the oligo were added separately (Figure 8A, *lanes 4, 6 and 8*), some spots were visible at the AFM in the open 6p120_6cF9 *DNA Origami* (Figure 8B, 7 and 8). In the closed form, some areas with higher height profile rather than spots are visible, which make hard to speculate about the presence of $H^5$ protein inside the DNA origami structure.

## CONCLUSION AND PERSPECTIVES

The results obtained at the ZMB in Germany are very preliminary, but aimed to establish new collaborations between our Italian group and the research group of Dr. Saccà. The effort to encapsulate a thermostable protein into a hollow and closed *DNA Origami*-based structure opened new activities and experiments, tuning the assembling conditions and/or using alternative DNA structures. In particular, in collaboration with a Saccà's colleague, Prof. Siebers, we are planning the preparation of a $H^5$-fusion protein with a thermostable esterase from *Sulfolobus acidicaldarius* (ORF Saci1105), testing its activity in an environment different from that physiological or from the classical *in vitro* conditions.

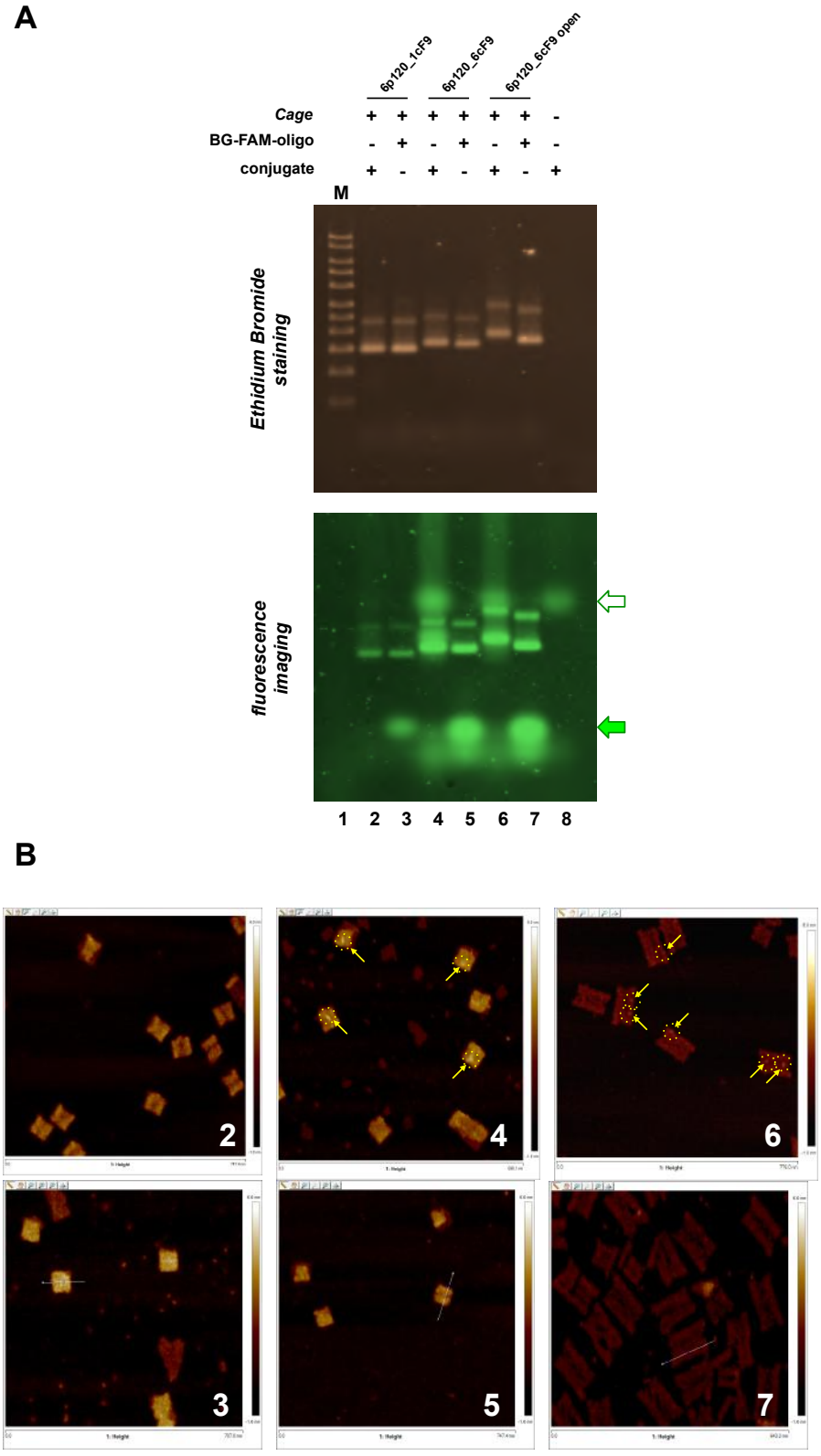


**Figure 7:** *Encapsulation of $H^5$-oligo conjugate in the DNA Origami cage*. (A) Agarose gel analysis as in Figure 6. Arrows as indicated in Figure 5. (B) AFM analysis of the samples, numbered as indicated in lanes of the gel in panel A. Yellow arrows and dotted circles indicate the presence of the $H^5$-oligo conjugate.

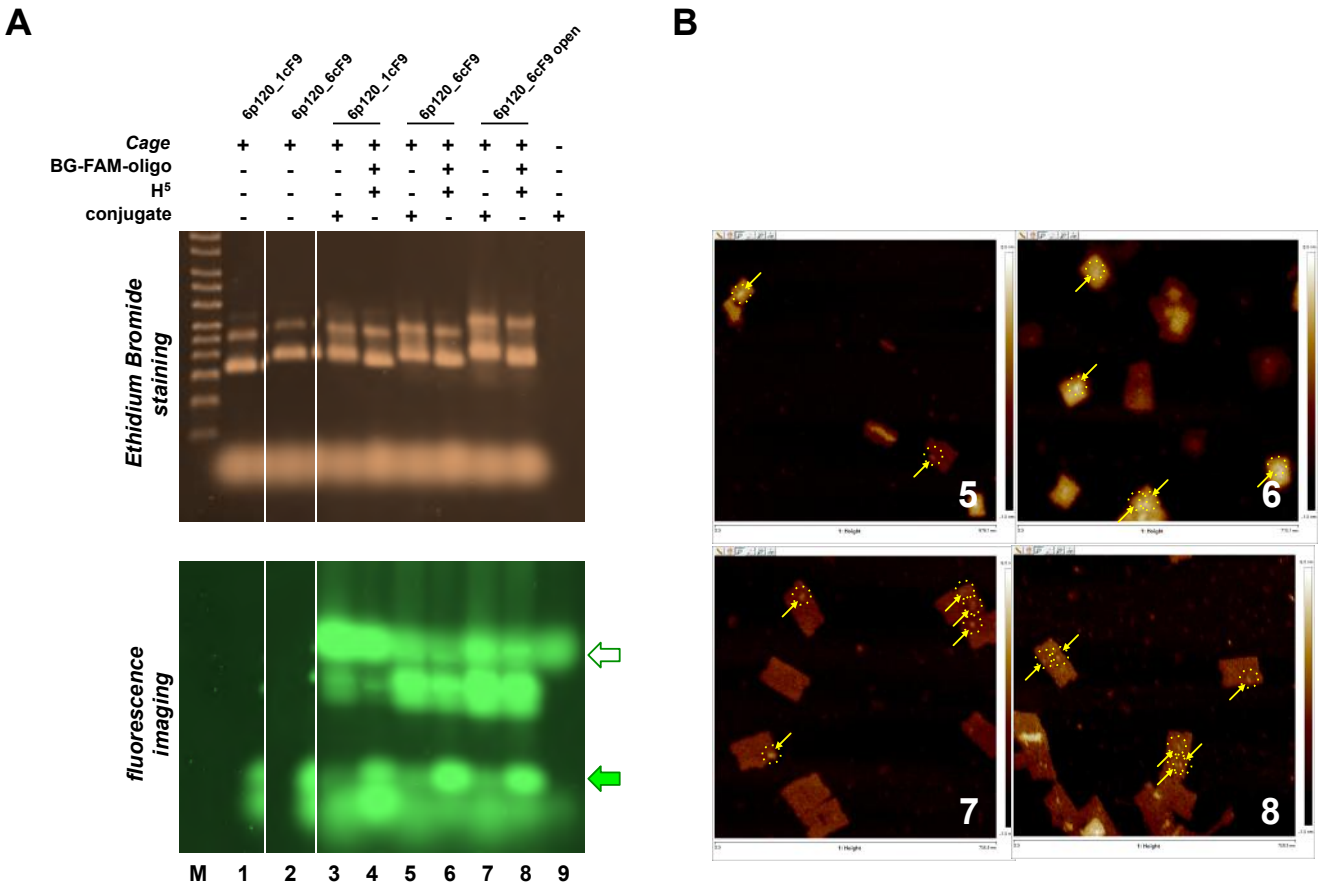


**Figure 8:** *One-step encapsulation of $H^5$-oligo conjugate in the DNA Origami cage.* (A) Agarose gel analysis as in Figure 7. Arrows as indicated in Figure 5. (B) AFM analysis of the samples, numbered as indicated in lanes of the gel in panel A. Yellow arrows and dotted circles indicate the presence of the $H^5$-oligo conjugate.

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