DNA-driven assembly of micron-sized colloids
Geerts, N.

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4 Clustering versus percolation in the assembly of colloids with long DNA

We report an experimental study in which we compare the self-assembly of 1 µm colloids bridged through hybridization of complementary single stranded DNA (ssDNA) strands (12bp) attached to variable-length double stranded DNA spacers that are grafted to the colloids. We considered three different spacer lengths: long spacers (48500 bp), intermediate length spacers (7500 bp), and no spacers (in which case the ssDNA strands were directly grafted to the colloids). In all three cases the same ssDNA pairs were used. However, confocal microscopy revealed that the aggregation behavior is very different. Upon cooling, the colloids coated with short and intermediate length DNA’s undergo a phase transition to a dense amorphous phase that undergoes structural arrest shortly after percolation. In contrast, the colloids coated with the longest DNA systematically form finite-sized clusters. We speculate that the difference is due to the fact that very long DNA can easily be stretched by the amount needed to make only intra-cluster bonds, and in contrast, colloids coated with shorter DNA always contain free binding sites on the outside of a cluster. The grafting density of the DNA decreases strongly with increasing spacer length. This is reflected in a difference of the temperature dependence of the aggregates: for the two systems coated with long DNA, the resulting aggregates were stable against heating, whereas the colloids coated with ssDNA alone would dissociate upon heating.

4.1 Introduction

The double helix of DNA consists of two negatively charged phosphate-sugar polymer chains held together by hydrogen bonds between complementary bases on each chain. This specific feature makes this biomolecule of great interest to materials science, since it allows for specific and reversible binding. This property of DNA is of particular relevance for the self-assembly of constructs that contain different sequences of single-stranded DNA (ssDNA). This property of ssDNA was initially used to detect specific DNA sequences or to determine mismatches within a genetic code [81, 82]. The selectivity of DNA hybridization has been exploited to build nano- and microstructures in solution [19, 20] and to guide the self assembly of DNA-derivatized structures [147, 148].

In this chapter, we focus on the properties of colloidal particles functionalized with ssDNA. Colloids coated with complementary ssDNA will exhibit a sharp aggregation transition upon cooling. This property was exploited by Mirkin and co-workers [3, 82]
who used the induced aggregation of gold nano-colloids to detect specific gene fragments. From a “materials” perspective, the selectivity of DNA hybridization offers the opportunity to design novel colloidal structures and materials [149]. The number of base-pair matches tunes the strength of the attraction between colloids functionalized with complementary oligonucleotides, but there are many other parameters that control the resulting interaction between the colloids: e.g., ionic strength, grafting density of hybridizing DNA strands and length of possible spacers connecting the “reactive” ss-DNA to the colloids. A number of experimental [4, 150] and modeling [49, 51, 93, 151] studies have focused on the effect of temperature on DNA-driven phase transitions in nanoparticle systems. A recent paper [69] reports a study of the effect of ionic strength and hybridizing strand concentration at constant temperature. However, relatively little is known about the influence of the length of connecting spacers on the phase behavior of DNA-coated colloids. Theoretical work of Tkachenko and co-workers [9] indicated that possible crystal structures of binary colloids coated with DNA should depend strongly on the length of the spacer. We note that it has turned out to be particularly difficult to make crystals of DNA-coated colloids [123]; most of the self-assembled structures observed thus far correspond to large, amorphous aggregates [5–7]. A possible factor might be that the effective range of attraction between colloids coated with short DNA strands is much shorter than the diameter of the colloids. From experiments on polymer-colloid mixtures [152], it is known that gelation often preempts crystallization when the range of attraction between the colloidal particles becomes short. If something similar would hold for DNA-coated colloids, one might expect that varying the length of the DNA spacers might influence the kinetics of crystallization. However, things are not that simple. In earlier work we used colloids coated with extremely long double-stranded DNA linkers that carry a short single-stranded DNA sequence at the free end [53]. We showed that this type of colloids aggregated to form finite-sized clusters, surrounded by a DNA “halo”. Interestingly, the colloids inside these clusters were found to be rather close, an effect that is presumably closely linked with the expulsion of DNA from the inside of the cluster. In fact, a theoretical study of Bhatia and Russel [142], showed that a pair of colloidal particles, linked by long, strongly bound telechelic polymers would attract at short distances under conditions where flat surfaces coated with the same polymers would repel. This therefore shows that the Derjaguin approximation that was employed in the theoretical work of Ref. [9], breaks down for spacers with a radius of gyration that is not small compared to the size of the colloids, and this fact may be relevant for both the stability and the kinetics of formation of possible crystal structures. In addition, the absence of DNA-mediated repulsion at short distances implies that colloids coated with long DNA may get into close contact, so much so that strong, short-ranged attractive forces may cause the aggregation to become irreversible upon heating the system above the DNA melting temperature $T_m$.

As was shown in Ref. [123], other stabilizing agents (for instance, the grafting of a dense, inert polymer brush) may be needed to make aggregation fully reversible in such cases.

In this chapter, we study the effect of varying the DNA spacer length on the aggregation behavior of DNA-coated colloids. The work of Bhatia and Russel’s suggests
that, as the size ratio of colloid to polymer exceeds one, the repulsion at short distances is recovered; the strength of the repulsion depends both on the size of the polymers and their grafting density. In addition to examining the behavior of λ-phage DNA, which has a radius of gyration ($R_g$) of about 800 nm, we studied colloids coated with a pBeloBac11 plasmid ($R_g \approx 200$ nm), leading to a colloid to polymer ratio of 1.6. The interesting feature of this plasmid is its cos-restriction site. With the use of an enzyme (λ-terminase) we can regenerate the same sticky-ends as with λ-phage DNA, making the length of double stranded DNA (dsDNA) the only difference between the two systems. Finally, we compare the behavior of colloids coated with relatively long dsDNA and short “sticky” ssDNA end with a system coated only with the short ssDNA, and with a system coated with DNA that has no “reactive” ssDNA end groups.

4.2 Results and Discussion

4.2.1 Colloidal assembly depends on the length of the DNA bridge.

To compare the effect of the length of the dsDNA polymer spacer, two batches of DNA-coated colloids were prepared. One batch contained the longer λ-DNA the other the shorter pBelo-DNA. For both batches, A and B colloids were mixed in equal ratios, a 20 $\mu l$ aliquot of which was displayed in an imaging chamber. Cluster formation was followed with time beginning $\sim$30 minutes after mixing the colloids. At room temperature both DNA-coated colloid batches follow the same aggregation speed in the beginning. Small differences begin to show after one day and become most apparent after one month.
clustering time.

We find that DNA-driven aggregation progresses in different ways for the two DNA bridges used. The longer \( \lambda \)-DNA yields finite-size clusters that appear stable after more than a day (Figure 4.1a). In contrast, we observe a system-spanning cluster containing most colloids in the system, of colloids coated with the shorter pBelo-DNA with 25 sticky arms per colloid (Figure 4.1b). Using the colloid coordinates measured in the confocal images, we employed a cluster analysis program (using a freely available script that can run on IDL-software) to generate a histogram of cluster sizes. In the cluster analysis, we start by determining the nearest neighbors of all colloids. Two colloids are deemed to belong to the same cluster if their distance is less than a preset threshold. The threshold distance depends on the size of the linking DNA: for \( \lambda \)-DNA we chose \( r_c = 4 \mu m \) and for pBelo-DNA, \( r_c = 2 \mu m \). We verified that the cluster size distribution was insensitive to the precise values of the cutoff. The resulting cumulative cluster-size distributions for \( \lambda \)-DNA are shown in Figure 4.2a. The ones for pBelo DNA are shown in Figure 4.2b. Cluster sizes were determined at five different time points, starting from \( \sim 30 \) minutes after mixing until one month of aggregation time. Cluster sizes first increase in time, but the cluster growth comes to a halt after one week when colloids are coated with \( \lambda \)-DNA. Small differences in the graph, compared to the curve of one month old clusters, are only due to statistics rather than a change in the sample. The cluster analysis shows that when colloids are coated with \( \lambda \)-DNA, only finite-sized clusters form. However, the cluster-size distribution is relatively wide: they range from a few colloids up to a hundred colloids. We note that both clusters with an equal number of red and green colloids and with an unequal number of red and green colloids are present. The assumption that all tethers are hybridized after more than a day indicates that the number of tethers per colloid is not fixed but, as expected, fluctuates around its average value of 10 DNA arms. In contrast to \( \lambda \)-DNA, pBelo-DNA coated colloids don’t aggregate to a finite sized structure. Figure 4.2b shows only a fraction of all clusters present in the sample after leaving it to aggregate for longer than a day. In fact, after a few days the size of a pBelo-cluster becomes system spanning. The presence of smaller clusters is a bit misleading in this case, as most of them are part of the same system spanning cluster, but connected to the rest by a branch that is outside the field of view of the confocal microscope. This becomes clearer when we image the same sample with an objective with a smaller magnification, (Figure 4.3).

The finite cluster sizes observed in the \( \lambda \)-DNA system is unexpected, in particular, because simulation studies by Starr and Sciortino [49, 51] show that percolating networks of ssDNA/nanocolloids are formed, even when the number of arms per colloid is on an average only 2.1, where 2 is the limiting value. However, in contrast to the short ssDNA used in those studies, we have additional long dsDNA spacers. Hence, a possible explanation for the shift from finite sized cluster to a system spanning aggregate is the length difference of \( \lambda \)-DNA and pBelo-DNA. \( \lambda \)-DNA is long enough for hybridization of all tethers between a pair of colloids; besides the hybridization of tethers on adjacent sides of the colloids, the \( \lambda \)-DNA can easily stretch to link the far side of one colloid to any complementary DNA on its partner. The energy penalty for this stretching should be only in the order of \( k_B T \), because the force it takes to stretch a polymer coil in good
Figure 4.2: Accumulative graph of cluster sizes of DNA-driven assembled micron-sized colloids. Aggregation is followed in time from 30 minutes until a month. Graphs are taken over at least four samples using a minimum of 5000 colloids. Data points shown are averages of 5 data points. A: Colloids grafted with λ-DNA. B: Colloids grafted with pBelo-DNA.

solvent is inverse proportional to its radius of gyration: \( F \sim k_B T/R_g^2 \). This means that clusters of λ-DNA coated colloids are relatively “unreactive”. In order for two clusters two connect, the pre-existing DNA bridges on both colloids should open and then reconnect in such a way that the clusters are linked. This is not impossible, but quite unlikely at room temperature, as the cos1/cos2 bond unbinds only at about 42 °C. At the same time, free sticky DNA ends can still diffuse fast enough to find all unbound counterparts within a cluster, thereby saturating all bonds. This would also be true, if the “valency” (number of binding DNA arms per colloid) of individual colloids differ from the average valency. This seems to be confirmed in the longer time measurements that show no further growth of the λ-DNA system after two weeks. In contrast, pBelo-DNA is too short of length to do the same. DNA on the outside of a cluster is less likely to link to a complementary DNA on the same cluster. As a result, the pBelo colloids have “dangling bonds” and are quite reactive when they meet another cluster. As a result, the aggregation process in the pBelo case only stops when effectively all colloids are attached to a single, large cluster. In order to strengthen our hypothesis that it is the length of the spacer dsDNA that drives the two different aggregation mechanisms, we also prepared pBelo samples with only about eight sticky DNA arms per colloid, while the remaining 17 pBelo arms were blunt (no sticky cos end). Long time measurements revealed the same percolation (Figure 4.4a) and cluster growth (Figure 4.4b) as with the ones with only sticky ends.

### 4.2.2 Temperature has no effect on clusters stability

As noted in Ref. [53], the clusters obtained in the λ-DNA system were extremely stable. Heating to 80 °C did not redisperse the colloids. Since the 12 bp “sticky ends” have a melting temperature of \( \sim 45 \) °C, we should expect the bridged DNA to dissociate at
Figure 4.3: Lower resolution confocal image of clustered colloids grafted with pBelo-DNA obtained after letting the sample aggregate for one month. Here several images, taken at the same focal plane, were assembled to get a better in-plane image of the percolating 3D-cluster. Scale bar is 50 µm.
4.2 Results and Discussion

Figure 4.4: The different aggregation mechanisms are driven by the length of the ds-DNA spacer rather than the number of “sticky” arms. A: Typical cluster obtained with pBelo-DNA coated colloids with eight “sticky” arms and 17 blunt dsDNA ends. Scale bar is 10 µm. B: Accumulative graph to compare cluster sizes between samples with 8 (black) or 25 (gray/white) “sticky” arms.

80 °C. As argued in Ref. [53], colloids coated with λ-DNA can approach each other close enough for strong Van der Waals forces to come into play, thus causing irreversible binding. Based on the calculations of Bhatia and Russel [142], we should expect some repulsion between colloids coated with pBelo-DNA. Indeed, we find that the A-B pair correlation shows a first peak that is about 200 nanometers away from close contact (data not shown). However, the repulsion is comparable to the thermal energy and therefore does not prevent irreversible binding. Hence, also for pBelo-DNA we find that the aggregates are remarkably stable and do not redisperse upon heating to 80 °C.

This inability to redisperse above T_m was already described previously [5, 6, 123]. We note that Ref. [123] also points to another possible cause of irreversible binding, namely interactions due to the proteins that are used to graft DNA onto colloids. Since the limited amount of DNA tethers per colloid leaves plenty of unbounded neutravidin on our surface, this can be a good explanation for the samples resistance to melting. This problem will be discussed in more detail in appendix A.

4.2.3 In the absence of specific forces colloidal assembly is negligible.

To test whether the aggregation process that we observe is the result of DNA hybridization between complementary “sticky ends”, we also studied the behavior of colloids coated by DNA that lacked a “sticky” ssDNA end. These colloids are covered by a polymer layer of the same length as pBelo-DNA. In the absence of any ssDNA, the DNA “mushrooms” should act as a repulsive barrier. Indeed, when mixed these colloids remain in monomeric form over a period of at least a month (Figure 4.5a). The absence of
Figure 4.5: Reconstructed 3D image of micron-sized colloids coated with DNA of similar length to pBel-DNA displaying no complementary single stranded cos1 or cos2 at the ends (blunt DNA). A: Monomers are stable in time. Scale bar is 10 µm. B: Pair correlation function of figure A.

any significant peak in the pair-correlation function belonging to this sample suggests a homogeneous stable solution (Figure 4.5b). This indicates that the non-specific protein binding and strong Van der Waals forces are depending on the DNA bridging to bring the colloids to a close enough distance.

4.2.4 Shielding colloidal proteins leads to cluster reversibility

Bare proteins can lead to non-specific binding. As our DNA-coated colloids are made from dilute solutions of DNA, the grafted DNA must be in mushroom conformation (i.e. swollen but non-interpenetrating coils) at the surface of the colloids. The DNA coverage is thus not limited by the neutravidin grafting density, but by the amount of DNA added and the space occupied by a “mushroom” of DNA. On the surface of the colloid numerous neutravidins are present with no biotin attached (there can be as many as 10^4 neutravidin per colloid (Invitrogen)). A polymer grafted on a surface at one point is free to move around. The energy costs for small movements are not high. As already described previously [53], during the aggregation process DNA gets expelled from the gap between two approaching colloids, leaving the protein surface “unprotected”. This can lead to non-specific binding and a strong Van der Waals attraction, resulting in a non-reversible system. To test if the “unprotected” proteins on our colloid surfaces are responsible for the lack of cluster reversibility we produced colloids with the single strands cos1 and cos2 directly bound to the surface leaving out the dsDNA spacer. Because these ends are very small compared to both our DNA bridges, they cover the colloidal surface with a dense brush of short ssDNA overhangs. This system forms large percolating clusters within 30 minutes (Figure 4.6a). The aggregation is very fast and is only “diffusion limited”, in contrast to the colloids with long DNA: there the number
of possible contacts per colloid pair is small and hence it is likely that aggregation is “reaction” limited. Leaving a sample of cos1/cos2-coated colloids at 80 °C for 1 hour redispersed all colloids to their beginning state (Figure 4.6b). Computing the pair-correlation functions for both temperatures shows a clear difference. The big cluster at room temperature has a distinct peak at 1 µm, indicating close contact (Figure 4.6c). The close contact is expected since the colloids (1 µm) are much larger than the short piece of ssDNA used (12 bps; 40 Å). The pair correlation function of the same sample at 80 °C is absent of any significant peak consistent with the redispersed colloids in the sample (Figure 4.6d).

Shielding all proteins on the colloids surfaces leads to a system that is reversible with temperature. This suggests that the stability observed with the longer dsDNA spacers is indeed due to non-specific interactions among proteins.
4.3 Conclusions

In this chapter, we report the aggregation in suspensions of three different kinds of DNA-coated colloids. All systems share the same single stranded 12 base-pair oligonucleotides as sticky ends but differ in the length of dsDNA spacer between the cos-ends and the grafting points on the colloids. The longest DNA spacer ($\lambda$-DNA) leads to stable finite-sized clusters, never exceeding 100 colloids. Reducing the length of the DNA bridge (pBelo-DNA) results in an increase of the number of DNAs per colloid and thus a change in valency (i.e., in the number of reactive ssDNA strands per colloid), leading to the formation of macroscopic aggregates. Moreover, by reducing the valency of the pBelo-DNA system such that it is comparable to the ($\lambda$-DNA system, while not changing the overall pBelo-DNA density per colloid, we observe percolation. This supports our assumption that the finite-size aggregation of the $\lambda$-DNA system is due to the saturation of all bonds within a cluster because of the large extension of the dsDNA spacers. Both systems of colloids coated by long DNA aggregate irreversibly, which is something that is not observed if the reactive ssDNA ends are passivated. This implies that the DNA bridging helps the subsequent irreversible binding. The aggregates thus formed remain stable well above the $T_m$ of the DNA.

In contrast, colloids coated with a dense brush of 12 bp ssDNA oligonucleotides aggregate readily but redissolve upon heating, indicating that the dense DNA-brush prevents irreversible binding due to short-range forces. In order to get a high surface density with long and flexible DNAs, a third component must be used. The DNA we used is too long to self-assemble in a dense brush on a surface in good solvot conditions; it will always go to a mushroom conformation. Stability can be obtained by covering the surface with a mixture of the long DNA tethers and shorter blunt dsDNA stabilizers (without “sticky ends”). Another option is to shift from protein binding to covalent binding of DNA to the colloids, although it seems likely that even then a third stabilizing component may be needed. The clear sensitivity of the kinetics of aggregation to the valency of the colloids indicates that for the controlled assembly of complex structures using ssDNA-coated building blocks experimental control of both the phase diagram and the aggregation kinetics will be of crucial importance and this in turn will necessitate the development of techniques to control the number of ssDNAs per colloid [49, 51].

4.4 Materials and Methods

4.4.1 Preparation of biotin-DNA solutions

Special care was taken to handle long $\lambda$-phage DNA (New England Biolabs) to avoid any damage due to shear. This DNA was pipetted with tips that were blunted and sterilized before use. The $\lambda$-phage DNA, predominately circular at room temperature, was linearized by heating in 25 $\mu$g/ml solution to 65 °C. Each linear dsDNA is terminated by two complementary 12 base single strands (ssDNA; “sticky ends”), which we call cos1 and cos2 (cos1 = 5’-GGGCGGCGACCT-3’; cos2 = 5’-AGGTCGCCGCCC-3’).

The linearized DNA was separated into two batches. One batch was mixed with a
solution (5 µl; 20 µM) of cos1-biotin oligonucleotides (Eurogentec), the other with cos2-
biotin oligonucleotides (Eurogentec). To hybridize the oligonucleotides to the DNA,
the solution was heated to 65 °C for ~30 minutes and then cooled overnight to room
temperature. Subsequently, T4 DNA ligase (New England Biolabs) was added to ligate
the DNA backbone. To remove the excess of oligonucleotides and enzyme the samples
were centrifuged and washed three times on a Microcon YM100 membrane (Milipore)
with Tris-HCl buffer (250 mM, pH = 8). The biotin-DNA solution was then recovered
in a clean tube.

The pBeloBac11 plasmid was purchased as a strain (New England Biolabs, ER2420S).
To obtain sufficient DNA for coating 50 µl colloids (1% solids), the strain was grown
overnight at 37 °C in 60 ml LB medium (for 100 ml: Bacto-Tryptone 1 g; Bacto-Yeast
extract 0.5 g; NaCl 1 g and ddH₂O to 100ml; autoclave to sterilize) in presence of
chloramphenicol (20 µg/ml). With a spin miniprep-Kit (Qiagen) the plasmid DNA
was then isolated. The purity of the DNA was checked by gel electrophoresis on a 1%
agarose gel. The plasmid contains the same cos-site that can be opened by restriction
with λ-terminase (BIOzymTC), leaving the same 12 base single strands (“sticky ends”).

As with the λ-phage DNA, the pBeloBac11 DNA was separated into two batches and
modified with biotin according to the same protocol described above.

4.4.2 Preparation of DNA-coated colloids

As in Ref. [53] we used a binary system in which DNA mediated attractions are favorable
only between heterogeneous colloids. Green and red fluorescent neutravidin-coated
polystyrene micro-spheres (diameter 1 µm, Molecular Probes) were functionalized with
DNA carrying complementary “sticky ends”.

Our conjugation protocol is based on the neutravidin-biotin coupling procedure de-
scribed in Ref. [53]: the two batches of λ-DNA or pBelo-DNA were separately mixed
with either green (“A” hereafter) or red (“B” hereafter) fluorescently labeled colloids,
coated with neutravidin, that were dispersed in TRIS-HCl (250 mM, pH = 8). In each
case the DNA and colloids were reacted overnight during which they were continuously
tumbled. The next day the samples were pelleted and washed five times to remove excess
of non-conjugated DNA. In between these washing steps, samples were heated once for
10 min. at 50 °C, to remove poorly bound DNA. The supernatant was retained to quanti-
fy (by UV-spectrometry at 260 nm) the amount of DNA that had effectively bound to
the colloids. For λ-DNA this leads to an estimate of some 10 DNA chains per colloid. We
prepared two types of pBelo-coated colloids, both with around 25 DNA grafted chains
per colloid. However, in one sample, all 25 pBelo-DNA chains carried sticky cos ends,
while in the other, only 8-10 sticky arms were present, with the remaining pBelo-DNA
being blunted. Latter samples were prepared with a ratio of $1/3$ of sticky end DNA and
$2/3$ of blunt DNA. Given the relative size of the polymer and the colloids, we expect
each colloid to be completely surrounded by grafted DNA. For thermo-reversibility tests,
we also prepared colloids, where the complementary ssDNA was directly attached.
The polystyrene colloids have a binding capacity of 4.5 nmol biotin/mg colloids, providing
20000-30000 binding sites for biotin-cos1 or biotin-cos2 nucleotides and thus a much
higher DNA coverage.

The DNA-coated colloids were diluted in a fresh TRIS-HCl buffer (250 mM final concentration, pH = 8) with D$_2$O to minimize sedimentation. This yielded two batches of colloids with either ds-λ-DNA (10 sticky arms per colloid) or ds-pBelo-DNA (either 8 or 25 sticky arms per colloid) grafted onto their surfaces by strong biotin-neutravidin bonds, displaying complementary single stranded cos1 and cos2 free ends respectively and one batch of colloids directly displaying the cos1 and cos2 free ends (no dsDNA spacer).

### 4.4.3 Preparation and coating imaging chambers

To avoid any nonspecific interactions between DNA-coated colloids and glass surfaces of the imaging chambers, coverslips were coated with polyethylene glycol (PEG). The coating of glass slides with PEG is a two-step procedure. First the glass slides were silanized with 3-mercaptoptriethoxysilane (97%, Fluka), then a 5-kDa maleimide modified PEG (Laysan Bio Inc) was tethered to the terminal thiol group of the silane.

Prior to the silanization, the glass slides were cleaned in an UV/ozone box. The cleaned slides were placed in contact with silane vapor overnight. Subsequently, the slides were rinsed with Ethanol, dried with Nitrogen and left in an oven (100 °C) for 30 minutes. Next a drop of concentrated maleimide modified PEG (250 mg/ml in TRIS-HCl buffer) is trapped between two silanized slides and left overnight at room temperature. The excess of PEG is removed by rinsing with ddH$_2$O.

A sample chamber is made from two glass coverslips of different size separated by a parafilm spacer (100 µm). The parafilm sealed the chamber on three sides. After loading the chamber with 10 µl of each population of DNA-coated colloids, reaching a final colloid volume fraction of $\phi \approx 0.004$, the chamber is completely sealed with glue.

### 4.4.4 Confocal imaging

A and B colloids were mixed in equal ratios, a 20 µl aliquot of which was displayed in an imaging chamber (colloids are suspended in a TRIS-HCl buffer (250 mM final concentration, pH = 8) with D$_2$O to minimize sedimentation). All suspensions were imaged at room temperature by means of an inverted microscope (DMIIRB, Leica) with a confocal spinning disc scan head (CSU22, Yokogawa Electric Corp.) and a 60x water immersion objective. Fluorescence of the two populations of colloids was excited at 488 nm and 512 nm. Emission was observed at 505 nm and above 600 nm respectively. The use of two types of fluorescent colloids and confocal microscopy allowed us to extract colloids coordinates and to reconstruct 3D images [2].

### 4.5 Acknowledgements

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