



Technologies for genomic and epigenomic analysis: a new frontier for micro- and nano-fluidics

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de Toulouse

Manuscrit présenté par

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En vue de l'obtention d'une

Habilitation à Diriger les Recherches de

Université Paul Sabatier

Technologies for genomic and epigenomic analysis: a new
frontier for micro- and nano-fluidics?

Soutenue le 28 octobre 2013 devant le Jury composé de

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1. Introduction

a- Where do we come from?

My scientific background is in physics with a major in fluid mechanics and chemical engineering. Lured by cross-disciplinary disciplines, I started my career in biophysics studying chromatin mechanical properties at the single molecule level. I acquired competences in microscopy techniques, microfluidics as well as in molecular biology. This research constitutes the foundation of my expertise, and it gave me a taste for developing new technologies that aim at gaining understanding on biological processes.

After my PhD, I performed my post-doc in cell biology at EMBL (Germany), which I chose to improve my degree of autonomy in biology, and to gain exposure to modern problems of life sciences. In 2006, I joined LAAS, which is a French stronghold for micro- and nano-fabrication. This laboratory was initially specialized in micro-electronics and in microsystems, two fields of research which had a spectacularly successful run of science and technology in the 90s. LAAS had to reinvent its research orientations towards nanotechnologies and biology to remain vital at the turn of 2000. At this time researches in microfluidics and in biodetection were initiated, and this activity is now one mainstream research direction. This work environment thus broadens my scientific spectrum to nanofabrication, and provides me technological solutions to face the modern race of innovation. This research direction has already been adopted by several leading edge professors in the US, who perform researches at the nexus of physics, biology and biotechnology. Moreover, in their recent white paper on “The convergence of life sciences, physical sciences, and engineering” (MIT, 2011), researchers from MIT posit that a revolution takes place at the nexus of disciplines, which will constitute a major source of innovation in biomedical sciences. Convinced by this new opportunity at the cross-roads of disciplines, and confident that the development of new technologies can further our understanding of biological transactions, I launched my own research activity focused on micro- and nano-systems for the analysis of chromosome structure and dynamics. This orientation is consistent with my training in chromosome biophysics. LAAS is now equipped for routine molecular or cell biology assays, but I also have to rely on a network of collaborators to perform more innovative experiments. This environment helps me to stay connected to the modern questions of biology, for I believe that this proximity is essential to develop relevant biotechnologies in the long term.

b- Where do we go?

My researches at the nexus of biology, fluidics, and engineering primarily rely on my background in fluid mechanics and in biophysics. The cutting-edge research environment of LAAS for micro- and nano-fabrication allows me to devise innovative miniaturized lab-on-chip devices for biodiagnostics and for fundamental biology. The spectrum of activities at the

frontiers of biology and technology is vast, and I am particularly interested by the pivotal role of technologies in the rapidly growing field of genomics and epigenomics.

In this manuscript my claim is that microfluidic and nanofluidic technologies offer great perspectives, in particular for their ability to manipulate minute samples down to the single molecule and single cell levels (Martini et al., 2007; Whitesides, 2011). I thus wish to confront micro- and nano-fluidic technologies to the modern questions of genomics and epigenomics. Among the long term perspectives of this research is the quest to devise analytical devices enabling to perform molecularly-detailed personalized diagnostics. Notably this long-term orientation is strengthened by the fact that chromosomes, which constitute an abundant material in cells that can be conveniently purified and manipulated for analytical measurements, is a biomarker of cancer, as we shall discuss below.

c- Objectives and overview on the manuscript

My research relies on a combination of competences in fluidics, engineering, physics, and biology. Any of these topics is addressed in wised and critical reviews, which are regularly published in specialized journals. Cross-disciplinary reviews are less frequent in the scientific literature, but they can be found in watch reports, which usually collect separate contributions covering a broad scientific spectrum. Because many authors contribute to these reports, the bridge between the different disciplines is not transparent, and the scientific questions that could be addressed by cross-disciplinary consortiums are not clearly asked. In this manuscript I opted for a format midway through the review and the watch report in order to show the relevance of combining fluidics, engineering, and physics to address modern questions in genome research, and to identify open scientific questions that can be investigated by cross-disciplinary technological developments. I am aware of the pitfalls of this exercise: the bibliography may not be exhaustive, and some ideas may be disputable and/or controversial. However, my hope is to produce a useful contribution for future researches.

The manuscript is divided into 7 sections, starting from a scientific overview on chromosomes (section 2), followed by a survey on the technologies for genomics and epigenomics (section 3). We then propose to describe our research in the framework of technological developments for DNA manipulation and separation (section 4), and chromosome biophysical properties (section 5). We then wish to overview the recent trends regarding the alterations of chromosomes and their link to cancer (section 6) in order to motivate some of our research orientations (section 7).

2. Brief historical perspective on chromosomes

Chromosomes are formed with chromatin, which is the nucleo-proteic structure present in the nucleus of eukaryotes. Chromosomes are formed of nearly equivalent amounts of DNA and proteins. Their physical large-scale description is among the first achievements of light microscopy observations in 1850, because they appear as separate entities during mitosis and meiosis. Chromosomes were then isolated as karyotypes, and modern karyotypic analysis of human chromosomes was demonstrated in 1956 (Tjio and Levan, 1956). Interestingly, the protein component of chromosomes (histone proteins) was discovered in 1884 by Albrecht Kossel (Kossel, 1884), years before the disclosure of the DNA structure by Watson and Crick (Watson and Crick, 1953). The building block of chromosomes, which is called the nucleosome, was unveiled even more recently in 1974 (Kornberg, 1974; Olins and Olins, 1974). Chromosomes molecular structure therefore appears as a recent discovery, which still remains the subject of intense research.

The functional role of the nucleosome initially appeared as a sink to compact the genetic material inside a micron-sized nucleus, and as a block against polymerase progression. The field of chromatin biochemistry gain considerable interest in the 90's as the polymorphic nature of the nucleosome was disclosed, this polymorphism mainly stemming from the existence of histone variants, histone post-translational modifications and DNA sequence structural features (Lavelle and Prunell, 2007). These local heterogeneities, in association with nucleosome distribution along DNA, are now known to give tunable properties to chromatin, allowing it to control a wide variety of biological functions (Lavelle, 2009). Moreover, the rich palette of covalent post-translational modifications of histones, e.g. acetylation, methylation and ubiquitination, which have been shown to be associated to numerous biological functions, has suggested the existence of a histone epigenetic code in 2000 (Strahl and Allis, 2000), which is heritable through mitosis and sometimes meiosis but not coded in the genome. This proposition attracted much attention in the community, and the field of epigenomics has been growing steadily ever since (red curve in Fig. 1). Interestingly though, the word "epigenetics" dates back from the early 40s, a time when Conrad Waddington intended to explain that genetic variations did not lead to phenotypic variations (Waddington, 1942), and nucleosomes appear to represent one of the major physiological substrate to inherit information for gene expression regulation without changes in the DNA sequence. To further this argument, we note that the essential role of chromatin plays for gene expression regulation is regularly exemplified by comparing the lengths of the genomes of the prokaryote *E. Coli* ($4 \cdot 10^6$ bp), which is not folded in chromatin, to that of *Homo sapiens* ($3 \cdot 10^9$ bp): despite the difference of 3 decades in length, they encode for similar number of genes. Thus, much of our genome does not encode for genes, and the regulation of gene expression is a key characteristic of our cells.

Above the nucleosome level chromatin architecture is far less characterized. In fact the debate on chromatin structure remains as lively as it was in the early days of the field (note that the green curve (chromatin structure) is steadily growing in Fig. 1), and it has been proposed that chromosome structure remains among the least well understood in biology (Kornberg and Lorch, 2007). Anyhow the common textbook picture usually describes nucleosomes as regularly distributed entities *in vivo*, their repeat length varying from 160 bp in yeast to 260 bp in sea urchin. This long range correlation suggests that chromatin folds in higher order structures (van Holde, 1989), which are primarily associated to the formation of condensed nucleosome arrays that still remains the subject of debate. Although 30 nm fibres have indeed been observed *in vitro* by electron microscopy for a long time (Olins and Olins, 2003), they were only hardly seen in interphase native chromatin, namely in CHO cells (Belmont and Bruce, 1994), and their exact structure remains strongly debated (van Holde and Zlatanova, 2007). At larger scales, chromatin is arranged in three dimensions (Cook, 1999; Göndör and Ohlsson, 2009), allowing for cross-talk between distant chromatin loci in *cis* or in *trans* that participate in large-scale expression regulation. In fact the existence of a large scale organization of chromatin and of chromosomes in discrete territories (Cremer and Cremer, 2001) suggested that the “nuclear architecture” was a key research domain to further our understanding of biological transactions (blue curve in Fig. 1). In addition cytological analysis of nuclei using DNA staining agents lead to the proposition that chromatin could be considered as a bi-partite structure, existing either in an open configuration accessible to transcription factors and potentially transcribed (so-called euchromatin), or maintained in a compact and inaccessible state (so-called heterochromatin). This description is an oversimplification, and recent high-throughput analysis of chromatin post-translational modifications lead to the proposition that chromatin architecture could be segmented into 5 different states (van Steensel, 2011), a classification probably more arbitrary than the bi-partite proposition!

The field of chromatin has remained vivid over 40 years because new concepts emerged regularly over time. For instance, chromatin structure was initially assumed to be rather static, based on the observation that nucleosomes are very stable entities over time, and their physical disruption from DNA requires high salt concentration (typically greater than 1 molar). In fact, the static nature of the nucleosome appeared to be a remarkable exception at the turn of 1990 (orange and pink curves in Fig. 1), and the advent of live cell imaging technologies directly showed that molecular interactions in the chromatin context were highly transient (Misteli, 2001).

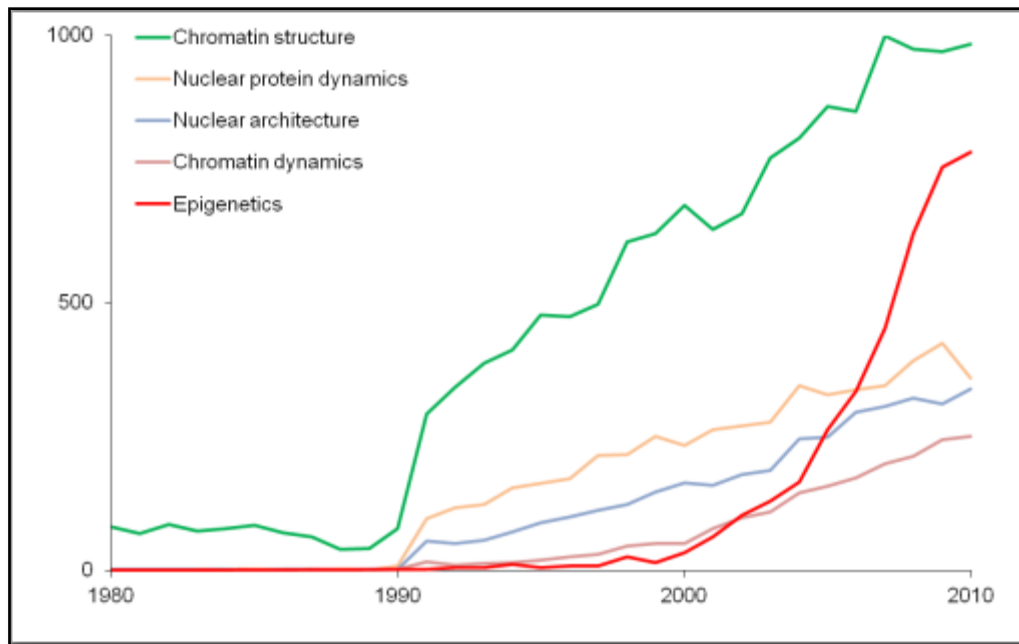


Figure 1 : Citation metrics according to ISI Web of Science for some of the most important concepts in the field of chromosome structure and dynamics.

During our research career, we studied the structure and the dynamics of chromosomes at different structural levels using a combination of techniques mostly invented by biophysicists. Before digging into our review on the main results obtained by biophysical methods, we wish to present an overview on breakthrough technologies that improved the physical and biochemical description of chromosomes at the genomic and epigenomic levels.

3. Technologies for chromosome analysis

a- The growing industry of DNA sequencing

Deciphering the genomes of virtually any organism is an old dream of biologists, which is becoming a reality. Using conventional molecular biology, in particular DNA modification, amplification, and size separation, two historical methods were developed by Maxam and Gilbert at Harvard (Maxam and Gilbert, 1977) and by Sanger at Cambridge (Sanger and Coulson, 1975) in the mid-70's to read the sequence of an unknown DNA fragment. These researchers obtained the Nobel Prize for their discovery in 1980.

The reliability of the Sanger process was rapidly selected for industrial developments, and the first automated sequencing automates were marketed in 1987 by Applied Biosystems (ABI 370). The key concept of this technology is to color-code genomic sequences using modified nucleotides called dideoxynucleotide triphosphates (ddNTPs), which bear base-specific fluorescent probes (see details in Fig. 2). These probes also stop the progression of the DNA amplification reaction, so that the output of a PCR amplification is a collection of DNA fragments of different sizes, each bearing a unique fluorophore at their 5' extremity. In the 90's the race to sequence the genome of organisms started, and the first complete genome of a living organism, the bacterium *Haemophilus influenza* (its chromosome contains 1,830,137 bases), was sequenced in 1995 (Fleischmann et al., 1995). The completion of the Human Genome project was then achieved in 2001 (Lander et al., 2001; Venter et al., 2001). Interestingly the Human Genome project was finished more rapidly than initially anticipated due to the considerable improvement of DNA size separation technologies and computer performances. DNA sequencing has now become a central tool in the panorama of diagnostics (Mardis, 2008): international networks are for instance organized for the genomic annotation of cancer vs. normal cells genome (The-Cancer-Genome-Atlas-Network, 2012).

At this point, it is interesting to draw a parallel between the meteoric rise of DNA sequencing with the evolution in micro-electronics over the past six decades. Indeed electronics constitutes an excellent example of transfer of academic research to the industry, leading to a global boost in innovation rate. This success has been initially formulated by Gordon E. Moore, who speculated in 1965 that the number of transistors that can be placed inexpensively on an integrated circuit would double approximately every two years (Moore, 1965). Moore's law leads to dramatic reductions in the cost and in the portability of e.g. personal computers, and it amounts for the fact that, once a technology becomes sufficiently mature to become industrial, its development is fast. The structural unit in electronic chips now measures 25 nm, and manufacturers will be able to produce chips on the 16 nm manufacturing process at the 2018 horizon according to estimates produced by e.g. the International Technology Roadmap for Semiconductors (ITRS), which is

a set of documents edited by a group of semiconductor industry experts. Clearly enough, this size reduction is not infinite, and the “the room at the bottom” of R. Feynman is starting to get crowded!

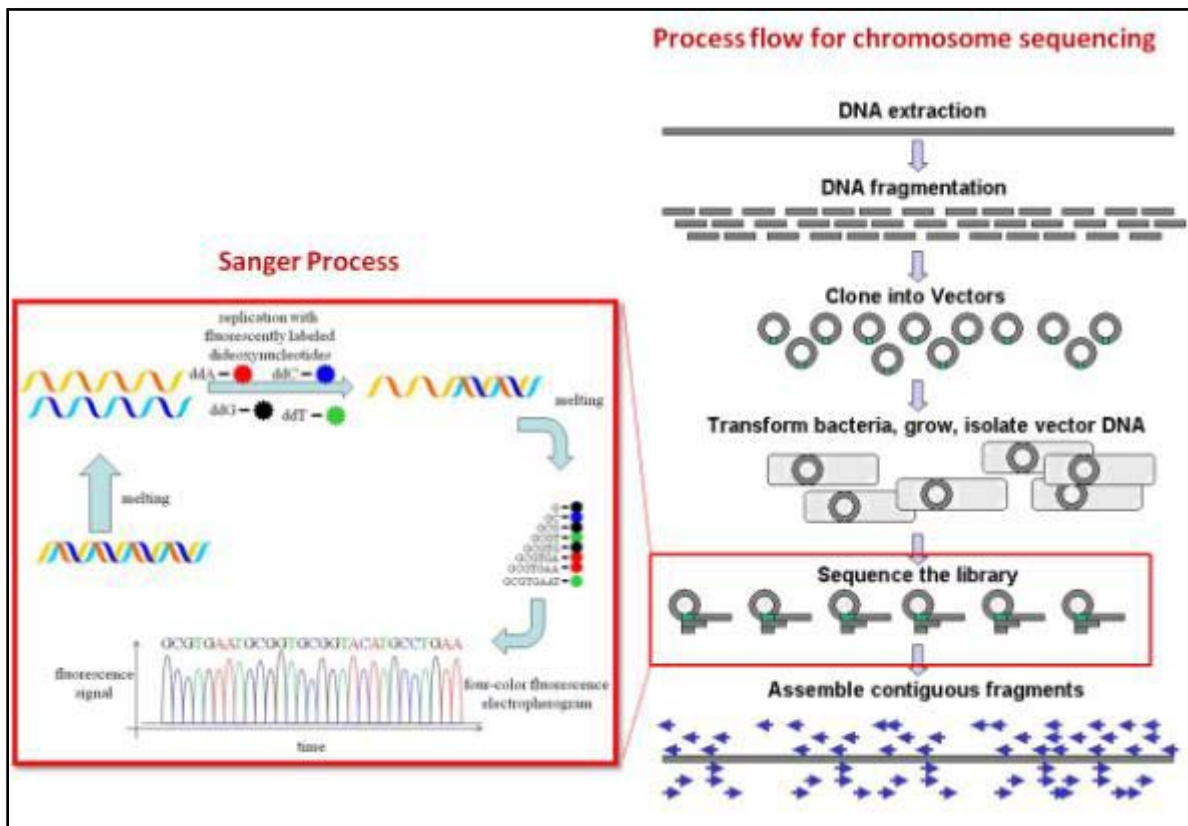


Figure 2 : The **left panel** represents the Sanger process, which consists in replicating a fragment of $\sim 10^3$ bp using a modified Polymerase Chain Reaction (PCR), in which color-coded ddNTPs are added in small amounts in the reaction mix (red, blue, black, and green). The replication reaction is stopped when ddNTPs are incorporated on the DNA fragments, and the product of the reaction is a population of DNA fragments of different sizes, which contain one and only fluorescent probe coding for the base at the 3' end. Size separation by gel electrophoresis allows to assign the genomic sequence based on the detection of a fluorescence signal (plot at the bottom). The **right panel** shows the process flow to decipher the genomic code on a chromosome. This technology is limited by several bottlenecks, namely the transformation of the vectors in bacteria, the sequencing of large vector library, and the registration of the clones on the chromosome.

DNA sequencing technology is now sufficiently mature to face the challenge of an industrial distribution, and this industry is exponentially growing, as shown by the exponential decrease of the cost to sequence one genome (upper left plot in Fig. 3), and as anticipated by Moore's law (Voelkerding et al., 2009). Interestingly, the emergence of next-generation sequencing devices in 2005 enabled to sequence an exponentially growing number of genomes, accounting the growing revenues of new companies (e.g. illumina which now employs more than 2000 people). This market is sustained by the dream perspective of highly personalized therapies, in which patients are treated with drug regimens that are specifically tailored to their disease (this dream is exemplified by the 2006 Science cover “Cancer treatment gets Personal” shown in Fig. 3).

Despite the justifiable excitement over human genome sequencing, however, this technology was already shown to be disappointingly inefficient to identify genetic components that account for known heritable traits or disease, leading to the “missing heritability problem” (Maher, 2008; Monolio et al., 2009). In addition the mutator hypothesis of cancer evolution, which speculates that genome caretaker genes are altered at the early stage of the disease (see more on this discussion in section 6.a), seems to be invalidated, raising serious concerns on whether sequencing provides enough information for molecularly-detailed diagnostics. Irrespective to this discussion on diagnostics, DNA sequencing has emerged as a key technology for academic research in chromosome structure and dynamics, as we will discuss slightly later in section 3.c.

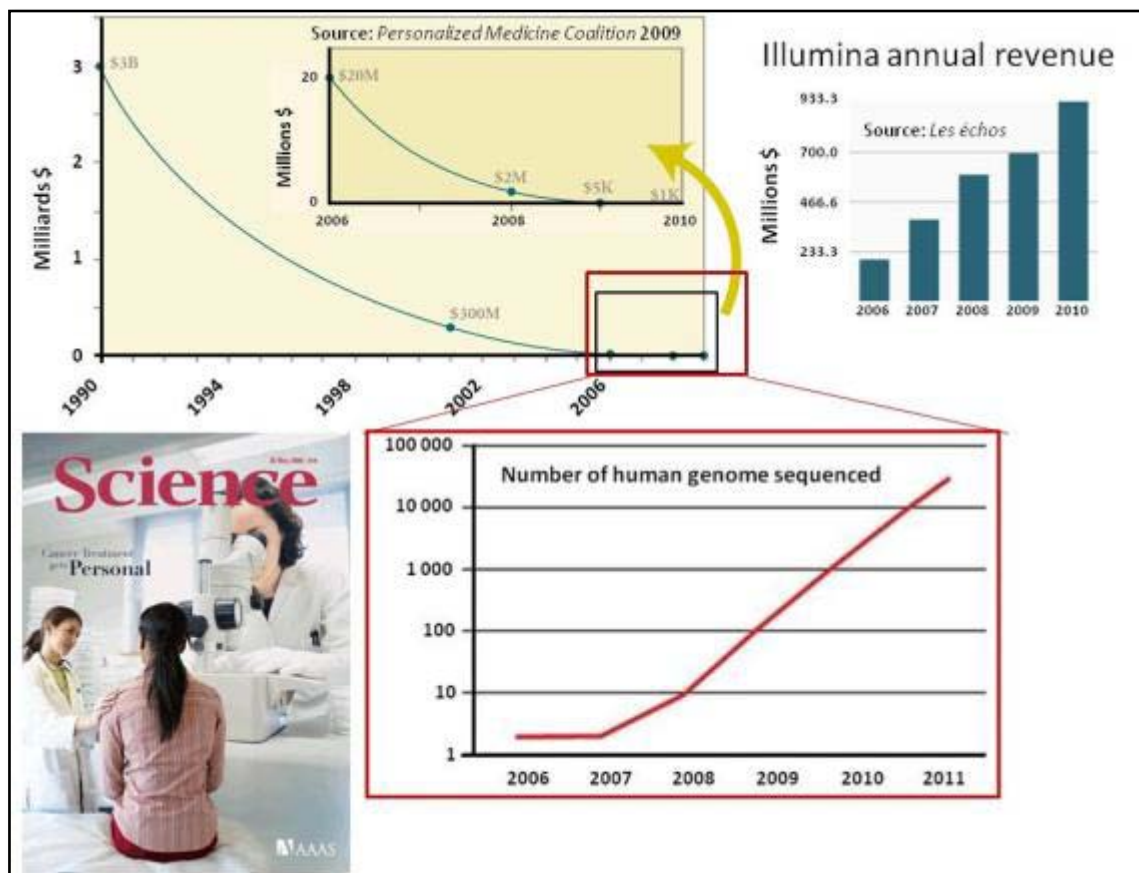


Figure 3 : According to the Personalized Medicine Coalition the cost of DNA sequencing is exponentially decreasing, and the number of genomes sequences each year is exponentially increasing. These trends account for the economic success of companies specialized in DNA sequencing (Illumina in the upper right plot), and to the idea of personalized medicine.

Importantly personalized treatments call for cost reduction, and the “holly-grail” is generally set to 1000\$ for one whole-genome sequencing. Technological efforts are still underway either based on reducing the cost of current “second-generation” sequencing automates, or on the development of “third-generation” sequencing technologies based on e.g. nanopores (OMNT Annual Report 2011, page 21), on physical manipulation of single DNA fragments using magnetic tweezers (Ding et al., 2012), which demonstrates the

conversion of the sequence in a mechanical signal with a minimal need for expensive reagents, or single molecule fluorescence detection for single molecule sequencing (Helicos Biosciences).

In parallel to these developments, huge efforts are consented to reduce the number of cells in samples analyzed by sequencing. Indeed, standard sequencing technologies generally enable to collect flattened data averaged over $\sim 10^4$ cells, and they may overlook crucial information for diagnostics associated to the increasingly acknowledged heterogeneity of cancer (Anderson et al., 2011; Navin et al., 2011). The variegation of cancer cells may constitute a roadblock to effective therapy if treatments are targeted to the predominantly detected subset of cells, which is not necessarily the 'stem' cancer clone. As for third-generation sequencing, different options have been tested for instance using conventional high-throughput sequencing starting from single cells deposited on 96-wells microplates with a dispenser (Navin et al., 2011), or by diluting the genomic content of 10 cells in 384-wells microplates in order to isolate one chromosome per well (Peters et al., 2012). In another direction, microfluidic networks have been developed to manipulate single cells, and isolate their chromosomes in separate chambers in order to collect the haplotype of single cells (Fan et al., 2011).

In conclusion DNA sequencing is still the subject of intense researches, bridging biologists and engineers in cross-disciplinary consortiums that aim to design innovative solutions to uncover the genome of limited subsets of cells at a very low cost.

b- DNA microarrays for transcriptomics and epigenetics

DNA microarray is also another breakthrough technology, which is now commonly used for molecular and cellular biology applications, and which has rapidly reached the level of reliability required for industrial development. It evolved from Southern blotting, which was invented in 1974 to identify DNA sequences in complex mixtures (Southern, 1974). Southern blotting consists in transferring DNA molecules on blot paper after their size separation by slab-gel electrophoresis, followed by sequence identification using fluorescently- or radio-labelled oligonucleotides. Note that Southern blotting also paved the way to Western and Eastern blotting, which are some of the most popular assays for the analysis of protein and RNA, respectively.

While Southern blots are performed on blot paper, DNA microarrays rely on the deposition of oligonucleotides on solid surfaces (flat or spherical in the case of particles), which serve as baits to capture their complementary DNA. On one single chip, arrays of oligonucleotides can be spotted either using conventional robotics or using *in situ* synthesis assisted by light lithography (Affimetrix process). The latter method only requires an efficient DNA grafting strategy (e.g. using thiol modified oligonucleotides on gold surfaces) and it is rather easily accessible to academic laboratories. However, it enables to generate low density chips containing up to a few thousands of probes. On the other hand the method

based on optical lithography is compatible with the fabrication of ultra-high density chips containing up to 2 million probes, but it is not adapted for long oligos (less than ~20 bp) because their synthesis is based on sequential light exposures, which induce damages to the DNA.

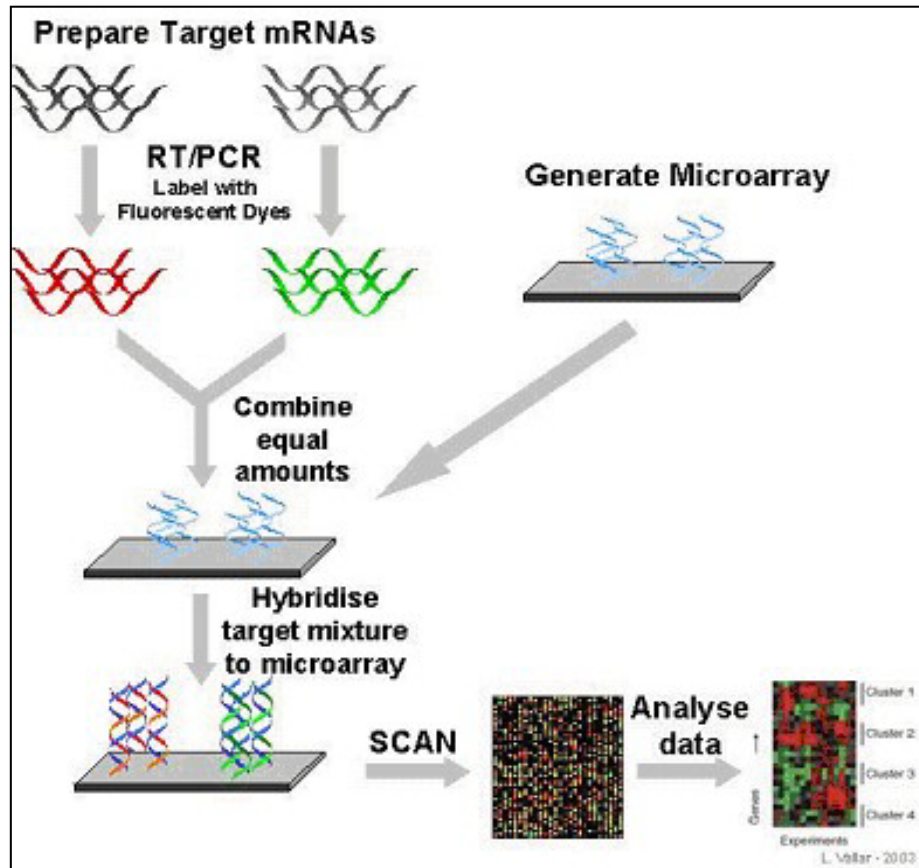


Figure 4 : Process flow for gene expression profiling on a DNA microarray. After purification of mRNAs from 2 samples, a reverse-transcriptase PCR is performed to convert RNA into DNA. In addition DNA is modified with an inorganic fluorophore for subsequent visualization on the microarray. The sample containing fluorescently-labeled DNA strands is titrated using a microarray using fluorescence scanning (Reprinted from Luxembourg Microarray Platform).

The first application of DNA microarrays was gene-expression profiling at the RNA level (Fig. 4, (Schena et al., 1995)). This process involves one step of reverse transcription to convert RNA into DNA followed by DNA hybridization on the microplate for subsequent analysis. This method then ushered in the field of transcriptomics, because it enabled to perform transcription analysis at the genome-wide level (Lashkari et al., 1997). Moreover the reliability of this technology was such that it was successfully applied for epigenetics studies, in particular for sequence identification after chromatin immunoprecipitation (see more below).

From a technological perspective, the development of DNA microarrays was associated to huge efforts aiming to set up protocols for biomolecules surface grafting. It is indeed important to insure a reproducible density of DNA, and to guarantee the functional

grafting. In addition the chip is composed of spots for DNA sensing separated by regions repellant for DNA adhesion. Despite nearly 30 years of efforts, quantitative DNA titration is still questionable with commercial devices: micro-array experiments are mostly based on ratiometric measurements comparing the fluorescence map of a control vs. target sample. Notably this ratiometric measurement is most conveniently carried out on one single chip by labeling the target and control DNA samples with spectrally-separated fluorophores (Fig. 4).

c- Assays for the analysis of chromosome structure and dynamics

Beyond the DNA level, a very wide body of techniques has been developed to investigate the structure of chromosomes. Rather than an extensive description of these methods (obviously a long and tedious task), we propose a classification, and discuss the impact and the field of application of each class. We figured out that the citation metrics of ISI Web of Science was a convenient (as disputable as it can be!) tool for this analysis (Fig. 5).

Conventional molecular biology techniques

Historical studies on chromatin were carried out using conventional molecular biology techniques: gel electrophoresis, Southern and Western blotting, electron microscopy, or analytical centrifugation, to name but a few. These techniques generally provide structural information on nucleosomes or nucleosome arrays.

Let us consider the following examples. The existence of nucleosomes was speculated based on results obtained by gel electrophoresis of DNA (band patterns electrophoresed after chromatin restriction using Micrococcal Nuclease), of proteins (discrimination by size of the four histones), as well as by electron microscopy (Kornberg, 1974; Olins and Olins, 1974; Oudet et al., 1975). The folding principles of nucleosome arrays was subsequently investigated using electron microscopy in combination with analytical centrifugation (Bednar et al., 1995; Bednar et al., 1998; Hansen, 2002a). In the 90s, molecular biology methods were adapted to investigate molecular interactions *in vivo* using chromatin immunoprecipitation (ChIP), which allows assessing whether a protein of interest is bound to a chromatin template by antibody affinity purification. This technology now occupies an ever-growing role in epigenomics (see below).

Biophysical techniques

Electron microscopy has just been referenced as a molecular biology technique, but it relies on electron microscopes, which are tools from physics. In fact, beyond the fact that our classification is somewhat arbitrary, light or electron microscopy techniques have always been rapidly adopted by the biology community most likely because they played historical roles in the description of cellular structures.

In the section, we propose to reference a few biophysical techniques that were used for structural characterizations of chromatin *in vitro*. Neutron scattering and X-ray crystallography are undoubtedly the most powerful technologies to unravel structural

information on nucleosomes at the atomic level, as exemplified by the key contribution in 1997 by Luger and collaborator who solved the atomic structure of the nucleosome core particle (Luger et al., 1997). Interestingly, their use has remained relatively limited over the years, maybe because atomic resolution nucleosome crystals turned out to be very difficult to form (note that the structure of Luger was obtained on a palindromic centromeric sequence). In another direction, the advent of single molecule technologies in 1995 (AFM or tweezers) that allow to control the conformation of a single DNA molecule in real time has attracted a lot of attention in the field of chromosome biophysics. The output of these techniques was not as fruitful as expected, because (i) chromatin tends to collapse on surfaces due to non specific interactions, leading to unreliable experimental data (this statement is based on our own experience of single molecule manipulation (Bancaud et al., 2006a)), and (ii) the structure of chromatin remains poorly understood so that the quantitative analysis of force-extension experiments is not univocal. As an example, it was concluded that chromatin folds in a zig-zag (Bancaud et al., 2006a) or solenoid conformation (Kruithof et al., 2009) using single molecule manipulation by magnetic tweezers (see more below on chromatin folding models).

Imaging techniques

Bright field microscopy techniques have been extensively used to visualize the nucleus and chromosomes since the early days of cell biology, for instance providing the description of chromosomes in 1842 by von Nägeli, the first models of nuclear architecture (Rabl, 1885), or the evidence of a bi-partite chromatin segmentation (Heitz, 1928). The field gained considerable interest with the emergence of fluorescence microscopy techniques, in particular confocal microscopy, which was invented by M. Minsky in 1957 and broadly commercialized in the early 90's. These imaging technologies shed new light on chromosome structure and dynamics in fixed or in living cells (black curve: fluorescence microscopy + nucleus).

To give a few examples, we should start with fluorescence *in situ* hybridization of fluorescent oligonucleotides (FISH), which was invented in the early 1980's (Langer-Safer et al., 1982), because this technique enabled to map with high spatial precision the position of genes in fixed cells. This technology was then improved to observe the organization of chromosomes in nuclei, showing their segmented repartition in discrete entities called chromosome territories (Cremer and Cremer, 2001). It also unraveled the concept of transcription factories based on the observation that the number of transcripts by FISH was much lower than the number of transcribed genes, thus hinting to the existence of transcription clusters (Cook, 1999). Despite these major contributions FISH suffers from its very low throughput, and from the fact that it was never applied convincingly to living cells (except for telomers (Molenaar et al., 2003)). The use of this technology is therefore no longer expanding (note the progressive decrease of the green curve).

In the mid 90's the advent of the Green Fluorescent Protein (GFP) and its fusion to virtually every proteins in living cells provided a new glimpse on the protein component of

chromosomes (red curve). Among other things, GFP labeling in conjunction with Fluorescence Recovery after Photobleaching (FRAP, blue curve), which infers relaxation dynamics in living cells, clearly established that molecular interactions in the nuclear context were highly transient with the notable exception of histones that remain stably bound to DNA (Beaudouin et al., 2006; Misteli, 2001).

As a final note, we wish to mention that electron microscopy has been extensively used to observe the nucleus, or to stain various nuclear structures. So far this technology met limited success because response of nucleosomes to electron beams is weak, so the resolution of observations is limited to large structures, e.g. heterochromatin foci. Nevertheless the race for high-resolution EM of chromatin is still very active.

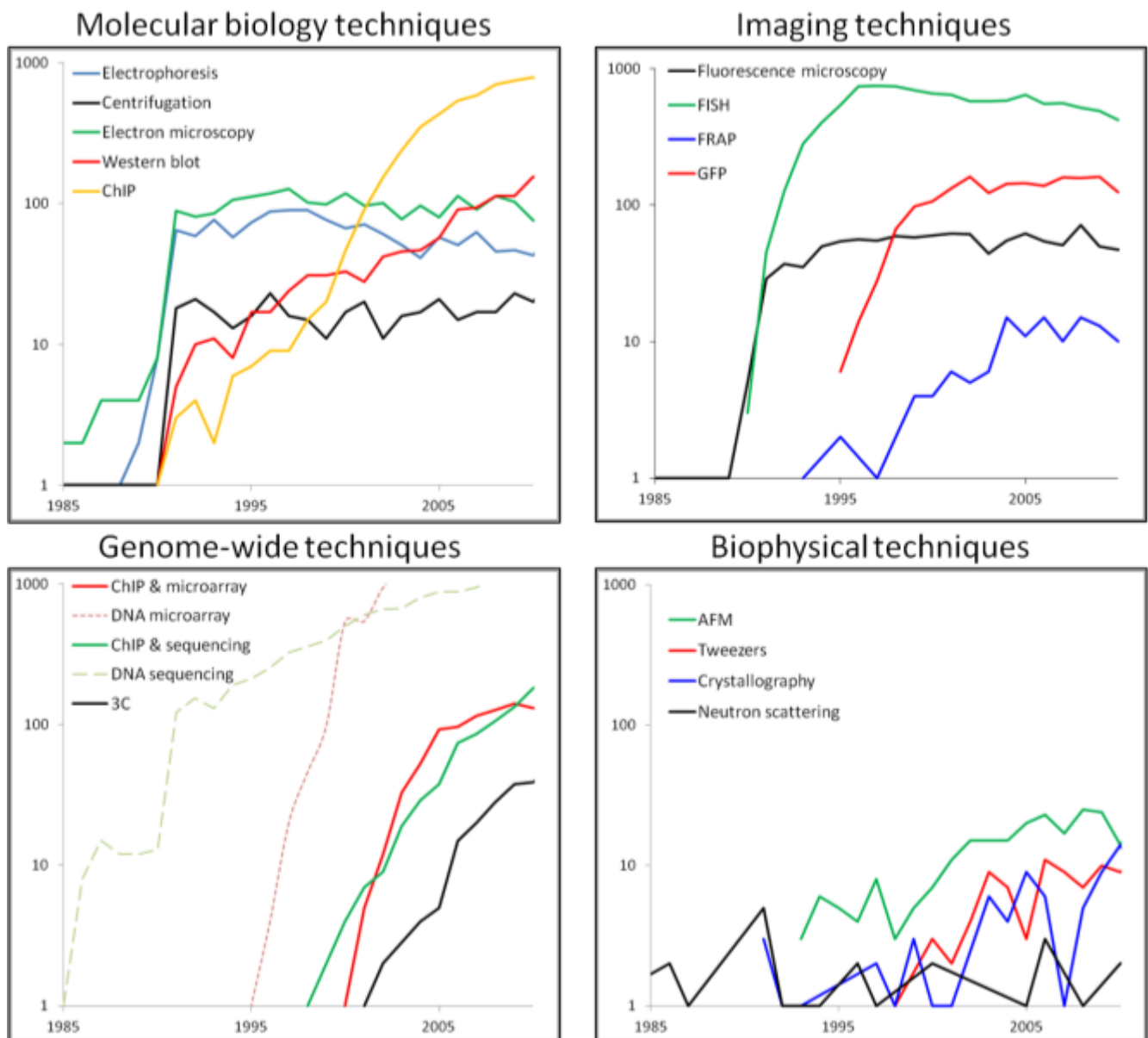


Figure 5 : Citation metrics according to ISI Web of Science for different technologies relevant to chromosome analysis. We chose to classify in four domains: conventional molecular biology methods (**upper left**), genome-wide techniques (**lower left**, note that the dashed lines correspond to the advent of microarray and sequencing technologies), imaging methods (**upper right**), and biophysical techniques (**lower right**). These keywords were

associated to “chromatin” or “nucleus” to obtain reliable values. Note that the search for the keyword “ChIP” in molecular biology techniques was performed without “sequencing” and “microarray”.

High-throughput molecular biology techniques

Over the last decade high-throughput molecular biology tools developed extremely rapidly, because they have the unique potential of mapping the distribution of epigenetic marks or molecular interactions in the genome. These technologies are based on ChIP, which allows to pull down virtually every protein of interest together with their binding sequences by immuno-precipitation, followed by high-throughput DNA sequencing or DNA microarray in order to retrieve the complete genomic information. This technological breakthrough is therefore the result of the convergence between conventional molecular biology and advanced commercial technologies. Among their outputs ChIP and sequencing enabled to map the localization of nucleosomes in budding yeast, showing that nucleosome positioning was to some degree encoded in the genome (Segal et al., 2006) through repulsion mechanisms based on the inadequate flexibility and intrinsic curvature of DNA for nucleosome accommodation (Miele et al., 2008). In a more general perspective, the success of the human genome project ushered in the idea of the international ENCODE program (Encyclopedia of DNA Elements), which aims at identifying all functional elements – in essence epigenomic elements – in the human genome sequence. The pilot screen describing 1% of the human genome was published in 2007 (Encode_Project_Consortium, 2007).

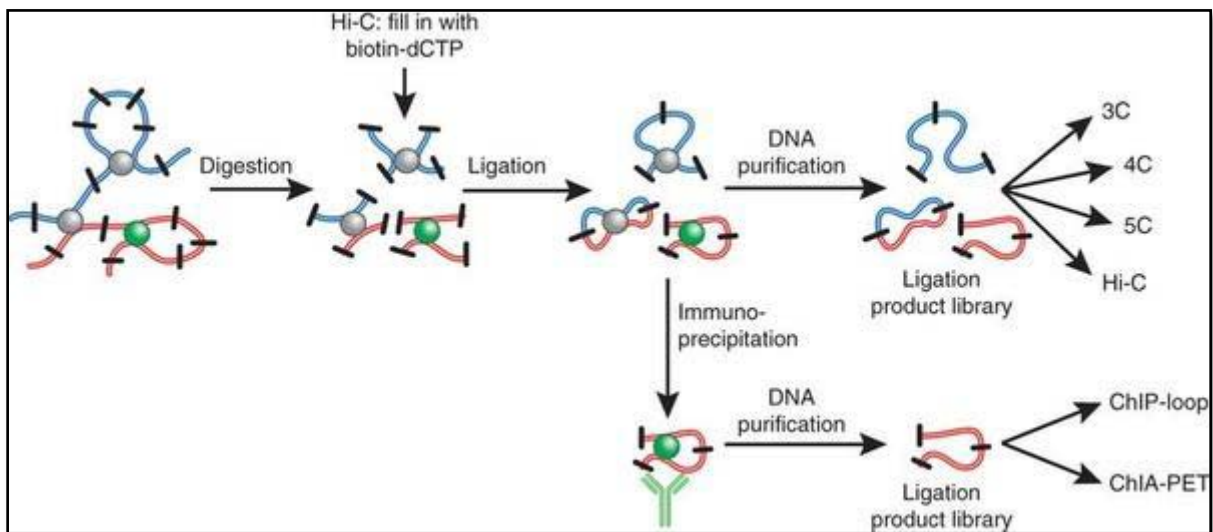


Figure 6 : Principle of chromosome conformation capture methods, and their combination with ChIP analysis for comprehensive genomic analyses.

In another direction, molecular biologists developed new tools coined chromosome conformation capture to map the contacts between chromosomes in *cis* and in *trans* ((Dekker, 2006; Dekker et al., 2002), and hence to elucidate the folding principles of chromosomes *in vivo*. Chromosome conformation capture is very similar to ChIP, for it relies on DNA-protein cross-linking, DNA restriction, followed by DNA ligation, and purification (Fig. 6). The resulting short DNA fragments are sequenced, and the library is compared to a

reference genome in order to determine the reads associated to a cross-link (red-blue segment in Fig. 8; (Lieberman-Aiden et al., 2009; van Steensel and Dekker, 2010)). Hi-C shed new light on the folding of chromosomes (see more below, (Lieberman-Aiden et al., 2009)).

d- Are there future technological breakthroughs?

Our technological overview shows that modern genomics and epigenomics rely on three essential technological breakthroughs, namely DNA sequencing, DNA micro-arrays, and fluorescence microscopy, which were invented in 1975 for the first two, and in the 30's, for the latter. DNA sequencing and Southern blotting emerged from conventional molecular biology, which has remained a very vivid science during ~50 years according to a timeline edited by the MIT ((MIT, 2011); Fig. 7). Both technologies were then brought to an industrial level, starting the Genomics revolution in 1986 (Fig; 7).

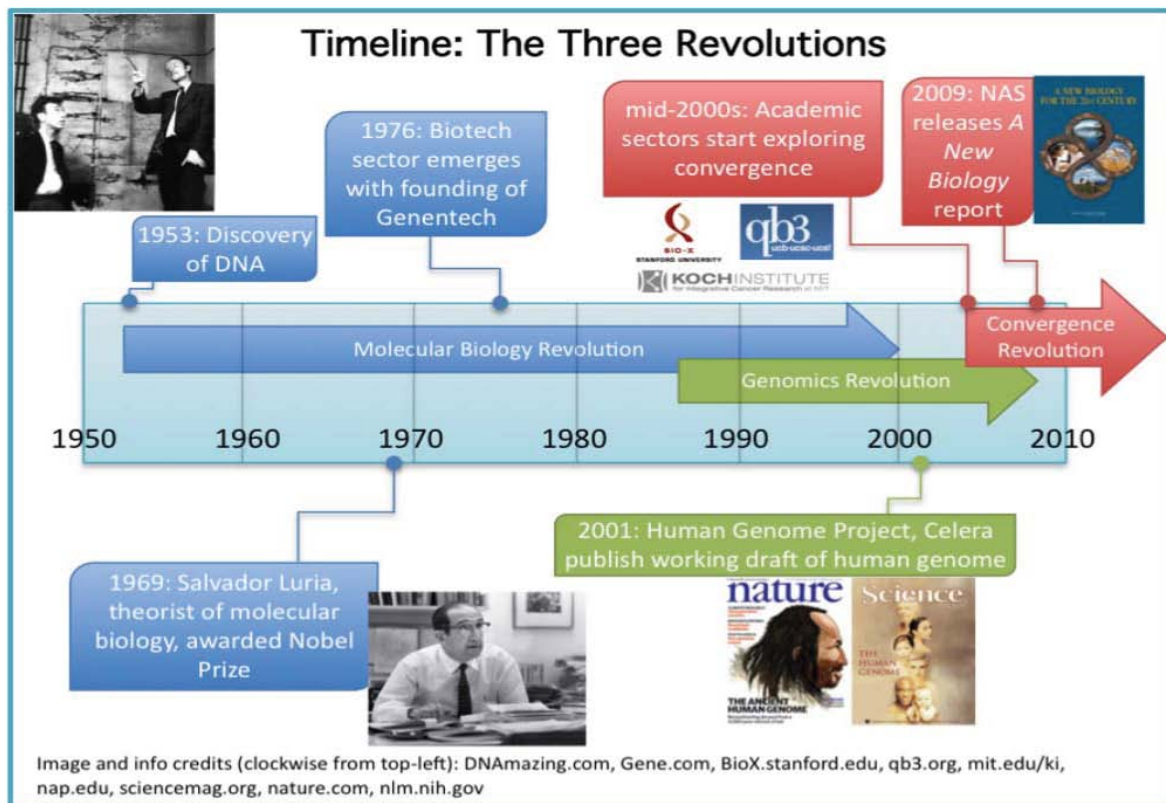


Figure 7 : Timeline proposed researchers from MIT in 2011, in which they speculate that the crossroad between biology, physics, and engineering constitutes a major source of innovation for biodiagnostics and biology.

According to the MIT report, the third technological revolution will involve joint efforts from biologists, physicists, and engineers and it will be driven by the needs for a safe environment, and for personalized diagnostics embedded in our daily environment. The roadblocks that impede this new revolution arise from the difficulty of federating cross-disciplinary consortiums.

Obviously this timeline has to be considered cautiously, but I am convinced that the convergence of technological developments is a huge source of innovation for biology and biomedical research. Fluidics, imaging, and engineering are some of the tools of this convergence, as we will describe in our research orientations.

We now wish to specify our technological orientations based on an evaluation of the built-in limitations of genomic and epigenomic assays. Let us introduce a “personal” representation of technological evolutions in genomics (Fig. 8). Each technology is represented by its DNA read length on the x-axis, and its throughput on the y-axis. For instance the first DNA sequencers commercialized by ABI could analyze DNA fragments of 10^3 bp at a low throughput of 10^3 - 10^4 bp/hour (blue ovoid in Fig. 8). Several boundaries can be defined in this graph: the genome size is $3 \cdot 10^9$ bp (vertical red dashed line), and the vertical dashed line corresponds to the sequencing of one genome in 1 hour. Also the average size of one human chromosome is $3 \cdot 10^9 / 46 \sim 5 \cdot 10^7$ bp (purple dashed line).

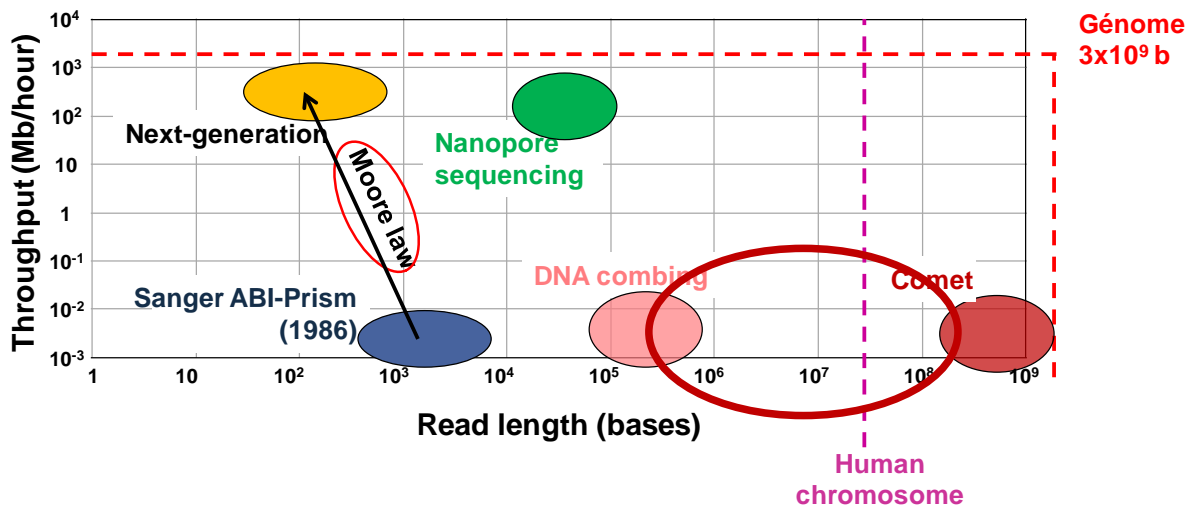


Figure 8 : Technological panorama in the field of genomic and epigenomic analysis. While next sequencing technologies typically use short DNA reads, we wish to analyse long DNA fragments in order to infer structural data at the whole chromosome level, and at the single cell level. Moreover, one of the main mission of technological researches is to improve the throughput of emerging technologies, and hence to move upward in our graph. The combination of high-throughput and long fragment analysis is our quest.

High-throughput sequencing machines read the human genome of ~ 3 Gbp in a few hours (orange ovoid, the black arrow shows Moore’s law direction). Surprisingly though this tour de force was achieved by reducing the read length to ~ 100 bp in comparison to ~ 1000 bp for historical machines. Such small reads are limiting in the power to detect sequence variation in the genome, based on their uniqueness. For example, if a given 32-base sequence is found more than once in the reference sequence, that sequence is unfaithfully represented. Unfortunately repeated genomic sequences (copy number variations) amount for $\sim 10\%$ of the human genome, and appear to play a critical role in cancer (Strankiewicz and Lupski, 2010). This situation for instance justifies the development of nanopore sequencing

(green ovoid), which aims at reading longer DNA fragments of $\sim 10^4$ bp in order to bypass the problems of repeated sequences.

A few assays were also developed to study genomic transactions at the chromosome or at the cellular levels. DNA combing (pink ovoid), which consists in spreading single molecules on hydrophobic surfaces using the forces exerted by a receding meniscus (Bensimon et al., 1994), enables to elongate chromosome fragments of 100-1000 kb on a coverslip and to observe these molecules with a fluorescence microscope (Fig. 9). This technology was particularly successful for studying the process of DNA replication at the single molecule level (Michalet et al., 1997). Note that the spreading process is based on capillary forces at the liquid-solid contact line, so that a large number of molecules can be manipulated simultaneously. This statement is however usually invalidated experimentally due to the limitations associated to the poor reproducibility of surface-biomolecule interactions, hence explaining our choice to set a low throughput for this technology. Interestingly, recent technological developments were conducted to increase the throughput of this technology using substrates engraved with micron-pits, which force individual molecules to spread at defined locations (Cerf et al., 2011).

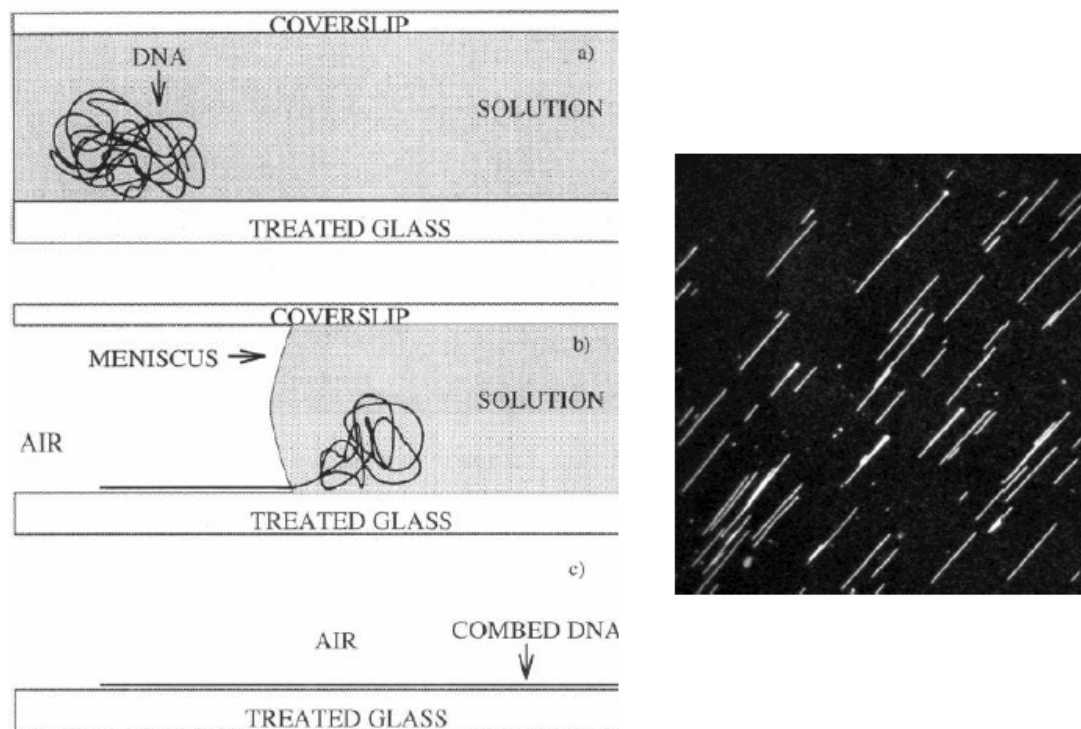


Figure 9 : The left panel shows the process to spread single DNA molecules by moving a meniscus. The right picture is a fluorescence micrograph showing a population of λ -DNA elongated on the surface (reprinted from (Allemand et al., 1997)).

At the cell level a few assays were developed to study genome alterations, mostly in the context of genotoxicity. The COMET assay (red ovoid), which consists in electrophoresing chromosomes extracting from single cells embedded in agarose gels, and in measuring the length of the chromosome comet after the migration (Ostling and Johanson, 1984), enables

to evaluate the number of single and double strand breaks in the genome (Brendler-Schwaab et al., 2005; Olive and Banath, 2006). This technology is also characterized by a low throughput due to the numerous steps for genome purification. As for DNA combing, microfabrication technologies have been recently used to increase the throughput of the comet assay (Wood et al., 2010).

Altogether, a consistent picture seems to appear. On the one hand, because the throughput of biology assays is low at their premises, technological efforts are accomplished to improve performances using “conventional” engineering based on well-established principles from microtechnologies, microelectronics, and optics. On the other hand technological researches should also seek for new functionalities. These functionalities should overcome some limitations of molecular biology assays:

- (i) they are reliable to manipulate short DNA fragments of $< \sim 20$ kbp, mostly because of the poor resolution of DNA separation matrices for high molecular weights¹. This situation explains the growing importance of bioinformatics: genomic information is collected in pieces that are registered by computers. Using this strategy, long-range correlations/interactions in the genome are however only indirectly sampled based on population averages;

- (ii) the number of techniques to characterize chromosomes is very limited;

- (iii) the techniques to characterize genomic transactions by immuno-precipitation generally involve large cell samples ($\sim 10^4$ - 10^8 cells for conventional ChIP analysis);

- (iv) imaging techniques are well suited to observe single cells with exquisite precision, and huge efforts are underway to develop platforms for high-throughput cell visualization in order to collect statistically significant data (Neumann et al., 2006). Conversely the manipulation of small numbers of cells is tedious with molecular biology tools, but reduction in size is one key challenge of modern genomics.

Consequently, **we believe that one relevant direction for future genomic and epigenomic researches consists in developing new technologies dedicated to the manipulation of whole chromosomes with resolutions of a 1-10 cells (red outline in Fig. 8). These systems should be combined to technologies for high-throughput cell manipulation and observation in order to screen heterogeneous conditions, and infer molecular mechanisms with single cell level resolutions.** This proposition is a meet-in-the-middle situation at the frontiers of imaging and molecular biology.

The recent evolutions in biology call for these innovations: the genomic and epigenomic heterogeneities of tissues have become increasingly clear over the past few years, and this characteristic has been suggested to one of the key contributor to this

¹ Long molecules are trapped in the the separation matrix, because the mesh size of agarose and polyacrylamide is 200-500 nm and 10-100 nm, respectively (see discussion in (Viovy, J.L. (2000). Electrophoresis of DNA and other polyelectrolytes: Physical mechanisms. Rev Mod Phys 72, 813-872.)).

problem. For instance, analysis of 100 single cells for breast tumors revealed three distinct clonal subpopulations (Navin et al., 2011), and the architecture of mutated genes in leukemia showed unexpected variegation, as inferred from fluorescence *in situ* hybridization (FISH) (Anderson et al., 2011). Interestingly, it has been suggested that this heterogeneity reflects the temporal expansion of tumors, thus providing invaluable information for clinical treatments. Further, the variegation of cancer cells may constitute a roadblock to effective therapy if treatments are targeted to the predominantly detected subset of cells, which is not necessarily the 'stem' cancer clone. However most molecular biology approaches enable to collect flattened data averaged over heterogeneous cell samples, thus potentially overlooking essential information. Therefore the specification of technologies for future molecular diagnostics requires for the **development of quantitative single cell assays**.

At this point, we wish to review some of the main results obtained on chromosome/DNA structure and dynamics obtained by biophysical techniques.

4. Contributions to the field of DNA separation and orientations

Electrophoresis is a central tool in bioanalytical chemistry for the separation of DNA, proteins, or hormones by size or charge. Size separation requires that two target analytes migrate at different speeds for a given electric field. Yet, this condition is not fulfilled for DNA in free solution because electric forces and hydrodynamic drags linearly depend on the length of the molecule (Viovy, 2000). As DNA separation is an essential tool of sequencing, huge efforts were consented to develop high-performance polymer matrices for DNA separation by capillary electrophoresis (Albarghouthi and Barron, 2000).

Polymeric separation matrices are intrinsically disordered systems, and Volkmuth and Austin developed the idea of separating DNA through “artificial pores” in 1992 (Volkmuth and Austin, 1992). These ideal matrices are composed of microfabricated 2D arrays of cylindrical posts, and they turned out to be particularly relevant for the separation of long DNAs of ~100 kbp (Bakajin et al., 2001), which are otherwise analyzed by pulsed-field gel electrophoresis during excessively long periods spanning several hours to days. Researches in artificial separation matrices have mostly been conducted with micron-scale obstacles (Bakajin et al., 2001; Duke et al., 1997; Randall and Doyle, 2004; Volkmuth et al., 1994), and the field attracted renewed interest with the emergence of nanotechnologies, which pave the way to the fabrication of nanopost arrays tailored from the molecular level to the macroscale (Dorfman, 2010).

In this panorama, we have conducted a few projects that we describe in the following. We then propose that new developments can be relevant in the context of genome research.

a- Self-assembled magnetic matrices for DNA separation

An elegant solution to generate reversible separation matrices consists in using the self assembly property of magnetic microparticles, which form hexagonal arrays of columns in slit-like channels in the presence of an homogeneous magnetic field ((Doyle et al., 2002), left panel of Fig. 10). The mesh size of these matrices can be tuned in the range ~2-5 μm by varying the channel geometry and the particle volume fraction (Minc et al., 2004). This mesh size is an order of magnitude greater than with agarose gels, so this matrix is relevant to the separation of long DNA fragments, as was demonstrated by the separation of λ - and T4-DNA in 150 s (49 and 160 kbp, respectively; (Minc et al., 2004)).

b- Nanopost-arrays and hydrodynamic actuation

The promises of nanotechnologies were confirmed experimentally, as some of the most impressive separation data were produced with ~250 nm and ~200 nm obstacles in radius (Kaji et al., 2004). In fact it is anticipated that DNA conformational variability during obstacle collision should be minimized with nanoposts (Dorfman, 2010), thus allowing to optimize separation efficiencies. In this rapidly moving research field, our recent

contribution consisted in revisiting the mechanism of DNA-nanopost collision using hydrodynamics instead of electrophoresis, which is the common actuation principle (right panel of Fig. 10). Though it has been speculated that these actuation forces produced similar responses, we demonstrate that the use of hydrodynamic flow fields to convey DNA molecules is associated to changes in the configurational space of hooking events, and to altered relaxation dynamics between consecutive collisions (Viero et al., 2011). This contribution is mostly interesting for the fundamental understanding of DNA-nanoposts interactions. Our feeling is that there is no breakthrough to be awaited for in this research, so we did not continue this project.

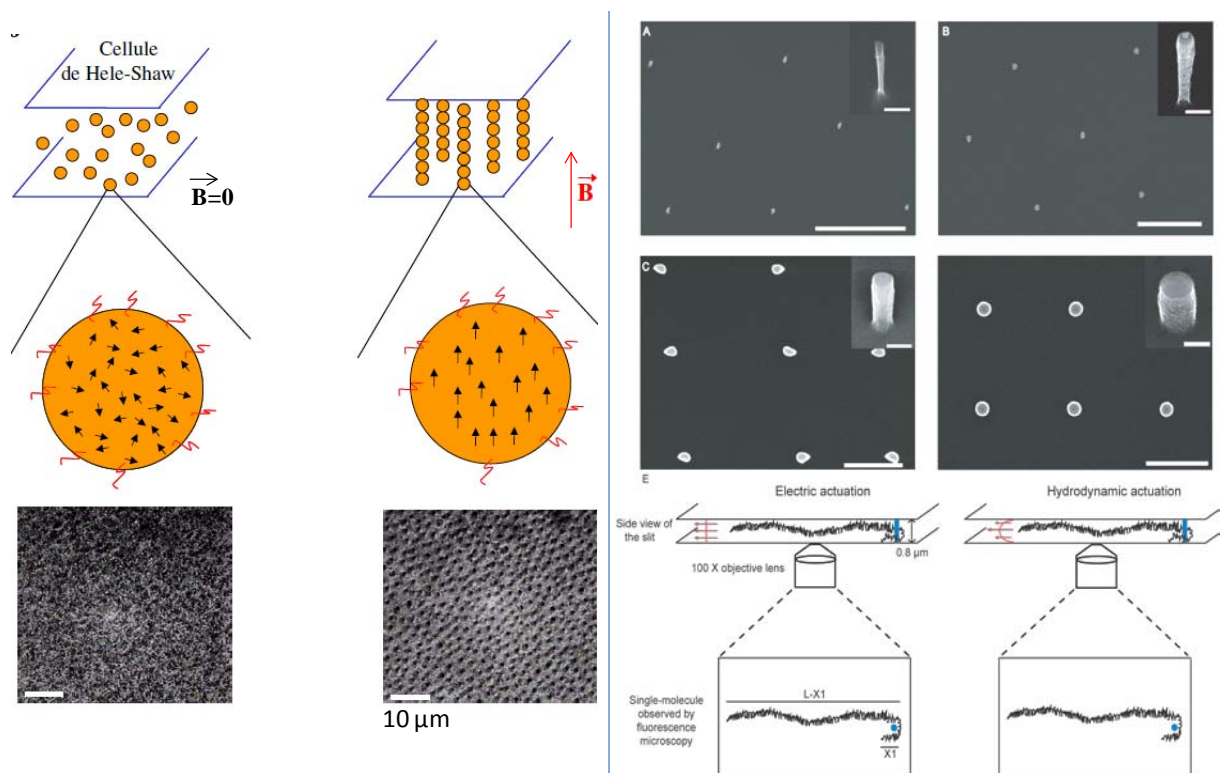


Figure 10 : (Left panel) Magnetic particles are placed in a slit-like microfluidic channel of 10 μm in height. Upon application of a vertical magnetic field, the dipole-dipole interaction favors their vertical alignment, and the repulsive dipolar forces between each column forces the formation of an hexagonal array. The typical dimension of the arrays are 1-2 μm , and the spacing 2-5 μm . (Right panel) Separation matrices can also be generated by dry etching of silicon, and this method enables to generate nanometric arrays of posts. The smaller posts measure 80 nm in diameter, and 800 nm in height. The scale bars of the pictures represent 1 μm , and 200 nm in the insets. We recently used these nanopost arrays as DNA separation matrices, and thoroughly compared hydrodynamic vs. electrophoretic actuation (Viero et al., 2011).

c- Matrix-free DNA separation

In parallel to the developments in artificial matrices, we noticed that good separation resolutions were obtained using matrix-free separation technologies. This approach was first described in a report by Zheng and Yeung in 2003, who used capillaries of 75 μm and observed the response of two DNA species of 48 and 5 kbp in the presence of cyclic and alternated electrophoretic/hydrodynamic actuations (Zheng and Yeung, 2003). These

authors detected a radial migration of the molecules toward the capillary centerline, the pace of which was dependent on the molecule dimension. Because the fluid velocity is greater at centerline of the capillary, the two DNA species migrate at different velocities, leading to size separation. This principle was then revisited by Wang and collaborators (Liu et al., 2011; Wang et al., 2012), who demonstrated the successful separation of DNA molecules spanning 10^2 to $5 \cdot 10^4$ bp using unidirectional hydrodynamics in ~ 60 minutes. They claim that the separation mechanism is a consequence of volume exclusion from the sidewalls, which increases with the DNA molecular weight². Notably this mechanism leads to a faster migration for high molecular weight molecules.

We recently fabricated slit-like channels spanning 0.5 to 5 μm in thickness, and applied electrophoretic and hydrodynamic flow fields simultaneously acting in opposite directions. Using continuous actuation we demonstrated that DNA molecules of $3 \cdot 10^2$ - 10^4 bp could be separated in a few minutes (Fig. 11). Very remarkably we observed a very sharp variation of the effective DNA mobility that is characterized by an optimal power-law scaling of -2.75. Separations are therefore much more efficient than with gel or capillary electrophoresis, for which the scaling is -1.

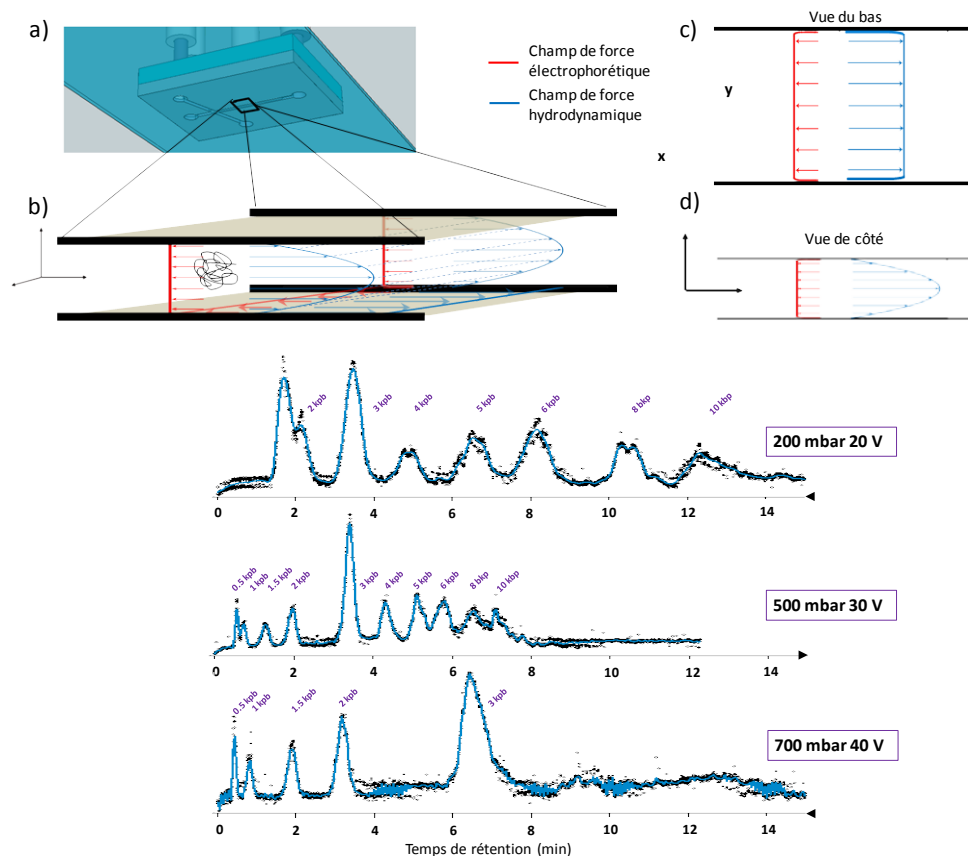


Figure 11 : (Upper panel-a) 3D representation of the microfluidic device with a slit-like geometry. **(b-c-d)** Pressure is applied to from left to right, generating a Poiseuille profile depicted in blue, and electrophoresis generates an homogeneous force field in the opposite direction (red symbols). **(Lower panel)**

² This model does not take into account any polymer physics, in particular the effect of shear stresses on the DNA conformation. We thus believe that they have not reached the endpoint of this research.

Electrophoregrams of the kb-ladder from NEB (experimental settings in the inset). Separation occurs in less than 10 minutes in a channel of 2 μm in height.

Although this research is still underway (a patent has been deposited in August), our working model is that the driving force of separation is the lateral migration of molecules associated to lift forces driven by hydrodynamic interactions of the molecule with the walls. Note that we are also exploring the contribution of asymmetric diffusion along the channel lateral dimension, which is associated to the difference in DNA conformation associated to the shear stress maximal near the walls and minimal at the centerline (Butler et al., 2007; Chelakkot et al., 2011). These mechanisms have only been proposed based on molecular dynamics simulations, so we set out to perform single molecule tracking experiments to confirm or invalidate this model.

d- What is our strategy?

We think that matrix-free DNA separation holds great promises for the manipulation of genomic samples for the following reasons:

- (i) the absence of separation matrix should enable to separate chromosomal DNA. Because this task remains a feat, we are testing this hypothesis with yeast chromosomes;
- (ii) the technology relies on a simple linear channel, which can readily be integrated in a fluidic network to perform multiple operations including in particular cell lysis and chromosomal DNA purification;
- (iii) the technology is readily adapted to the manipulation of minute samples comprising limited numbers of cells.

Overall, this assay seems to comply with the technical requirements to extract molecular information out of minute cellular samples.

5. Overview on chromosome structure and dynamics

In this section we describe the different structural elements of chromatin using the textbook representation, which assumes a segmented organization with DNA (~ 2 nm), nucleosomes (~ 10 nm), nucleosome arrays (~ 30 nm), loops (~ 200 nm), and chromosome territories (~ 1 μ m). Though this description is clearly an oversimplification (see e.g. the discussion on the fractal models in section 4.d), the corpus of data produced in chromosome biology takes this representation as implicit reference. **We mostly focus on the biophysical literature**, allowing us to introduce our contributions in this field.

a- The DNA level

In this section, we overview some of the main results obtained by single molecule techniques. We first focus on tweezers, which can be considered as the conventional set-up for DNA manipulation. We then show that micro- and nano-fluidics offer an interesting alternative for high-throughput data collection. Finally we describe recent results obtained by biophysical techniques concerning the profiling of epigenetic marks stored on DNA.

Manipulation by tweezers: DNA mechanics, and its structural polymorphism

The advent of single molecule techniques in 1992 (Smith et al., 1992) was a breakthrough in molecular biology because these techniques allowed to probe molecular transactions acting on DNA in real time. Moreover, the devices uncovered the underappreciated effects of tensile forces on biological reactions. Different devices, the most popular being optical tweezers, magnetic tweezers, and atomic force spectroscopy (Fig. 12, see (Bustamante et al., 2003) for a review), were conceived to control the conformation of DNA and sense its response to mechanical or biochemical cues. After 15 years of research, these systems allow exerting forces spanning 0.1-100 pN with a precision of a few %, detecting changes in contour length of a few nm with acquisition rates of ~ 1 s, and controlling the topology of DNA with a single turn precision.

Among the first achievements of tweezers techniques is the characterization of the elongational response of DNA, which was accounted for by the Worm-Like Chain (WLC) model (Marko and Siggia, 1995). The WLC describes DNA as a line that bends smoothly under the influence of random thermal fluctuations, and it relies on one adjustable parameter, namely the persistence length of the polymer ξ . The value of ξ defines the distance over which the direction of this line persists: correlation between the orientations of two polymer segments falls off exponentially (with decay length ξ) according to the length that separates them.

The persistence length of DNA in physiological buffer is 53 nm or 160 bp (Bouchiat et al., 1999), meaning that ξ is much larger than the polymer radius (~ 2 nm) and the Debye

length (~ 1 nm in physiological conditions³). Local heterogeneities in DNA structure associated to its genomic content were never convincingly shown to induce variations in elastic response in single molecule experiments, because DNA fragments of ~ 10 kbp are usually manipulated. Note that DNA is much more rigid than single stranded DNA ($\xi \sim 3$ nm (Tinland et al., 1997)) due to the stacking of the base in the double helix (Calladine et al., 2004).

The persistence length in torsion $C \sim 80$ nm was also measured directly by single molecule techniques based on the characterization of the response of DNA to topological constraints (Strick et al., 1996; Strick et al., 1998).

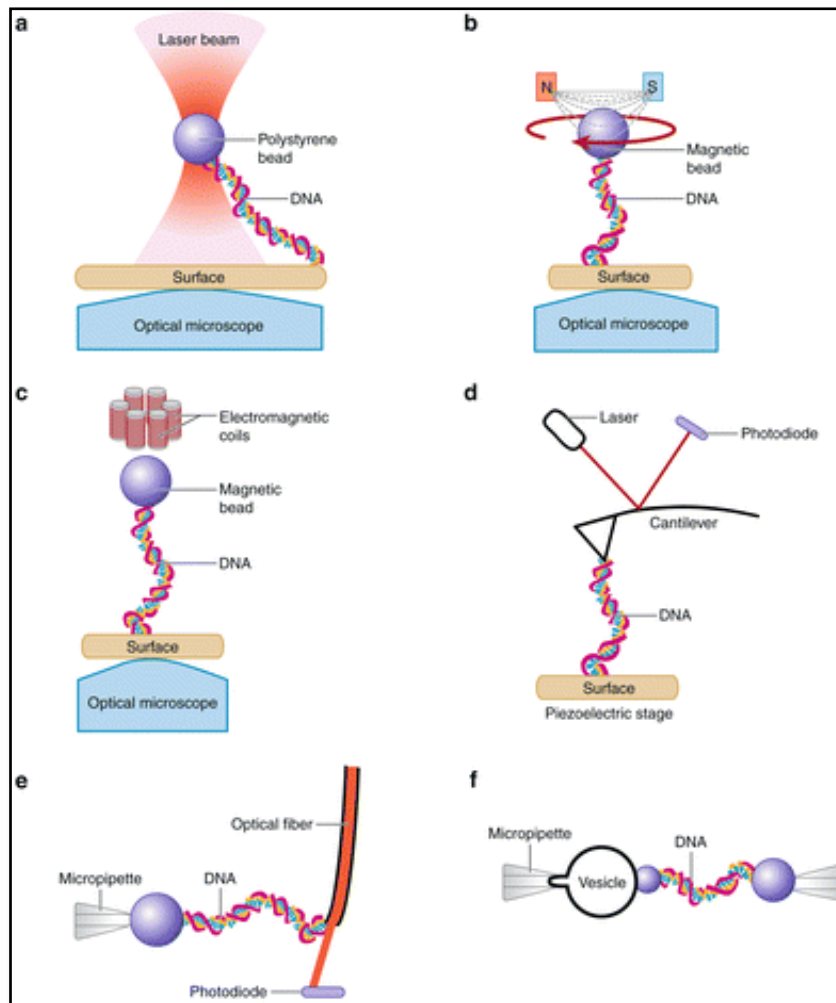


Figure 12 : This panel represents the most popular single molecule techniques to probe the response of DNA under mechanical constraints (force and/or torque). Reprinted from (Neuman et al., 2007).

³ The Debye length describes the length over which electrostatic interactions are screened. Its expression is $\lambda_D = (2\varepsilon_0\varepsilon_R k_B T / e\rho_0)^{0.5}$ with ρ the concentration of ions in solution, e the electron charge, $k_B T$ the thermal energy, and ε_R the electrical permittivity. For a detailed discussion on the Debye layer in the context of polyelectrolytes, refer to Barrat, J.L., and Joanny, J.F. (1996). *Theory of Polyelectrolyte Solutions. Advances in Chemical Physics XCIV*, 1-66. and Viovy, J.L. (2000). *Electrophoresis of DNA and other polyelectrolytes: Physical mechanisms. Rev Mod Phys* 72, 813-872.

Moreover, new conformations of DNA were disclosed by single molecule techniques: the exertion of forces greater than ~ 50 pN induced an unexpected transition to an elongated state, in which DNA length is 1.7 times longer (Cluzel et al., 1996; Smith et al., 1996). This conformation was coined S-DNA. Interestingly, the structure of DNA in the nucleo-filament formed with the bacterial recombinase RecA closely resembles that of S-DNA (Takahashi and Nordén, 1994), hinting to a functional role of S-DNA for recombination. Furthermore we showed that the binding of RecA is strongly enhanced on stretched DNA using single molecule experiments (Fulconis et al., 2004; Leger et al., 1998), suggesting that conformational fluctuations play a role in the binding of RecA to DNA.

Similarly, upon application of large positive torsional constraints, DNA was found to adopt a new conformation called P-DNA characterized by tightly interwound phosphate backbones and exposed bases (Allemand et al., 1998). Indications on the relevance of P-DNA are still lacking, and researches with proteins of thermophilic archaea, for which positive supercoiling is a hallmark, may add new lines to this story.

Microfluidics to increase the throughput of tweezers

Although single molecule techniques have reached amazing detection performances, they suffer from their low throughput: it is generally difficult to collect more than ~ 10 events per molecule. Many strategies have been developed to overcome this limitation, and we propose to review a few technologies.

Shear flow DNA manipulation

We improved a device pioneered by Ladoux and colleagues (Ladoux et al., 2000) to manipulate single DNA molecules in shear flows (Bancaud et al., 2005). This microfluidic system consists in attaching biotin end-labeled DNA molecules to streptavidin-coated surfaces, and in applying a controlled hydrodynamic flow field to elongate the molecule (Fig. 13A). The driving force of this elongation is the shear component of the flow, which is characterized by the shear rate $\dot{\gamma} = \delta v_x / \delta y$ following the reference defined in Fig. XXA (Doyle et al., 2000)⁴. This technology enables to simultaneously observe a large number of molecules on the field of view of a microscope, and it was successfully applied to characterize e.g. the chromatin assembly reaction (see more below, (Wagner et al., 2005)). Its main drawback stems from the fact that the constraints applied to the molecule are inhomogeneous, being null at the freely-floating end of the molecule and maximal at the anchor. Interestingly, Greene and collaborators used nanotechnology to generate controlled barriers on surfaces (Fig. 13B), enabling them to evenly distribute the molecules on the surface, and achieve high densities of ~ 1000 per field of view (Granéli et al., 2005). More

⁴ The parameter governing the response of single DNA molecules is actually the Weissenberg number, as defined by $\dot{\gamma}\tau$ with τ the longest relaxation time of the polymer in the Zimm or the Rouse regime depending on the experimental conditions. This definition implies that the deformability of DNA molecules increases with their size for a given hydrodynamic constraint.

recently, these authors developed a method to tether both ends of each molecules in order to elongate the molecule with a constant tension (Gorman et al., 2009).

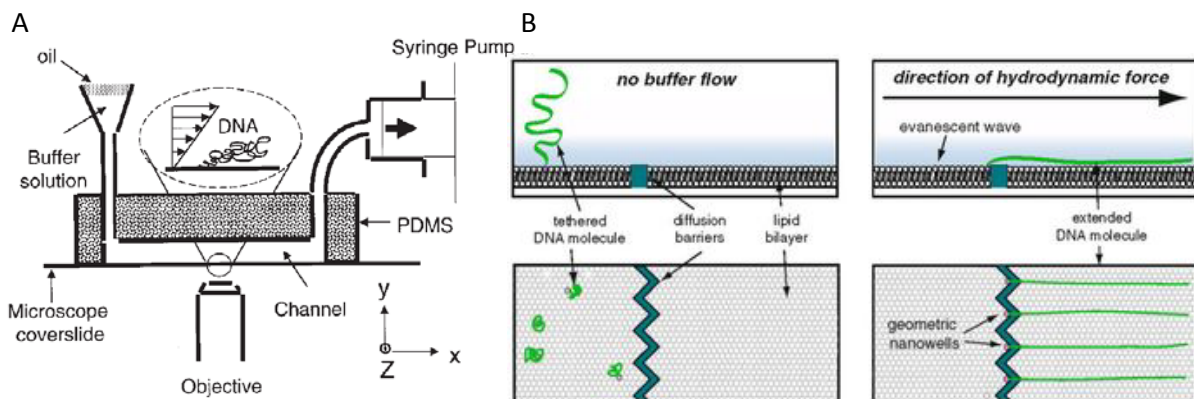


Figure 13 (A) Representation of the experimental set-up for DNA shear flow elongation. The fluorescently labelled DNA molecule is tethered by one end to the surface, and elongated using an hydrodynamic flow fluid in a microfluidic channel of $\sim 100\ \mu\text{m}$ in height. The device is placed on an inverted microscope. **(B)** The position of the molecules can be registered on the microscope coverslip using nanopatterned chromium barriers, and by tethering DNA molecules on lipid bilayers. This strategy allows to maximize the density of molecule in the microscope field of view (reprinted from (Gorman et al., 2009)).

Elongational flow DNA manipulation

Molecules can also be extended in elongational flows, which are characterized by longitudinal variations in fluid velocity, as described by the rate $\Gamma = \delta v_x / \delta x$ according to the reference defined in Fig. 14A⁵. The DNA fraction located ahead in the flow migrates faster than at the rear, leading to intramolecular tensile forces that elongate the molecule. This method allows to reach high degrees of elongation (Smith et al., 1999), in fact much greater than with bulk shear flows, in turn accounting for the fact DNA molecules manipulated in shear flows are tethered to surfaces. Different geometries can be envisioned to generate an elongational flow. On the one hand, the company U.S. Genomics has developed and marketed a high throughput DNA elongation platform for microbiology applications using a funnel geometry (Larson et al., 2006). This technology however suffers from the fact that DNA elongation is transient, and the extended conformation is somewhat variable, depending on how the molecules enter in funnels. On the other hand elongational forces can be exerted using the cross-flow geometry, which is characterized by a stagnation point ((Perkins et al., 1997), Fig. 14B). In this case, the trap is also transient but the escape time can be long because the trap is symmetrical. Although this method also suffers from limitations associated to the variability of the extended conformation, it was successfully applied to map the position of genomic targets on a long DNA template with a precision of 3 kbp (Zohar et al., 2010).

⁵ The parameter governing the response of single DNA molecules is the Deborah number, as defined by $\Gamma\tau$ with τ the longest relaxation time of the polymer in the Zimm or the Rouse regime depending on the experimental conditions.

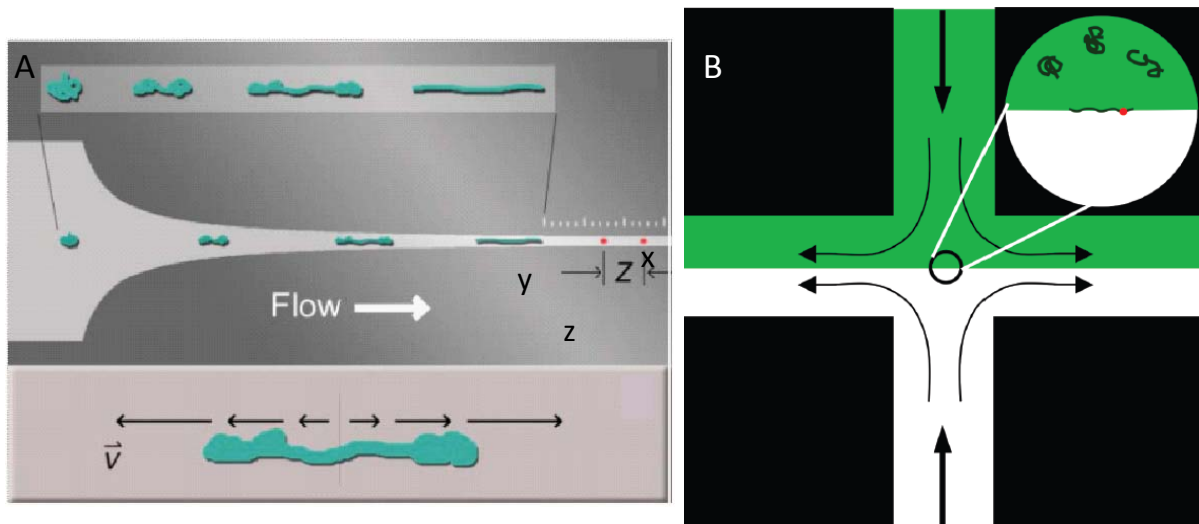


Figure 14 : (A) Representaion of a microfluidic channel with a funnel geometry. It produces an elongational flow that transiently extends the molecule (green structure). The lower panel shows the velocity field, as seen by the molecule during its migration in the funnel (reprinted from (Larson et al., 2006)). **(B)** Elongational flows can also be produced with cross-flow geometry. At the center of channels, the flow velocity is null, and the molecule remain transiently trapped in an extended state (show in the inset; reprinted from (Zohar et al., 2010))

Nanofluidics to increase the throughput of tweezers

Nanotechnologies enable to fabricate nanochannels of ~ 100 nm in cross-section. As DNA molecules enter in these structures, they undergo entropic constraints associated to the confinement of the walls, which force DNA molecules to spread longitudinally, as was demonstrated by Austin and colleagues (Fig. 15; (Reisner et al., 2005)). These authors also proved that the degree of elongation could be tuned by adjusting the nanochannels geometry, in turn showing that confinement in nanochannels could serve as a single molecule manipulation device. This technology holds great promise for high-throughput single molecule manipulation because arrays of thousands of nanochannels can be fabricated with parallel processes. Further the manipulation of single molecules is dynamic, and monitored by e.g. an electric field that can be tuned by an external operator in real time. A start-up company BionanoGenomics was developed to take advantage of these unique features in 2006.

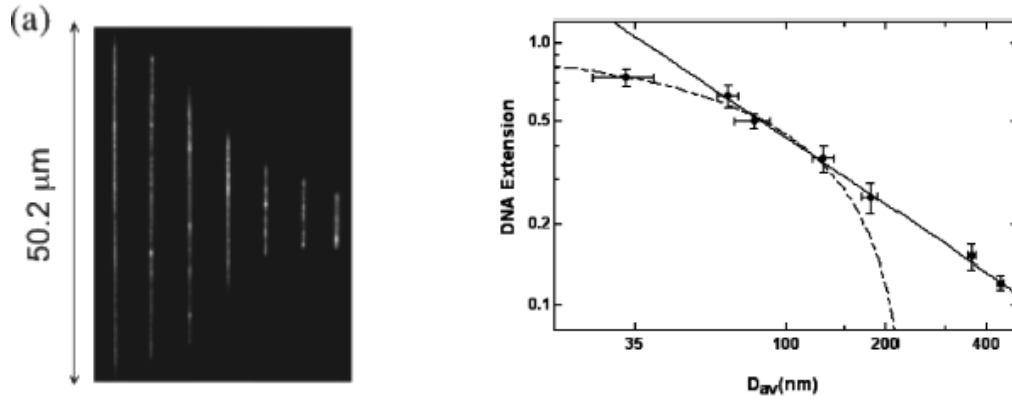


Figure 15 : The fluorescence micrograph on the **left** shows the spreading of single T2 DNA molecules in nanochannels of cross-dimension spanning 30 to 440 nm: the spreading increases with the degree of confinement. **The right panel** recapitulates experimental data of the normalized extension (l/L) vs. the nanochannel cross-section D , showing the quantitative agreement between the predictions (Eq. 1-2) with the experiment (Reprinted from (Reisner et al., 2005)).

Notably, in contrast to the microfluidic techniques listed above, the elongation is constant over time, so long as the molecule remains trapped in nanochannels. This steady extension enables to measure DNA lengths with an optimal precision of 400 bp using acquisitions of 1 minute (Riehn et al., 2005; Tegenfeldt et al., 2004). This precision is improved by a decade in comparison to microfluidic systems. In addition, the physics governing the spreading of DNA molecules in nanochannels has been investigated quantitatively, and two regimes with a cross-over at ~ 100 nm (that is, twice the persistence length) have been described:

- for a moderate confinement (the cross-section of the nanochannel is small compared to the gyration radius of the molecule but large compared to its persistence length), the elongation follows the de Gennes law (de Gennes, 1979):

$$\frac{l}{L} = \left[\frac{w\xi}{D^2} \right]^{1/3} \quad (1)$$

with L the contour length of the molecule, w the molecule diameter (~ 2 nm), and D the section of the nanochannel.

- the response of DNA departs from this behavior for $D \sim \xi$ (Odijk, 1983) according to:

$$\frac{l}{L} = 1 - 0.361 \left[\frac{D}{\xi} \right]^{2/3} \quad (2)$$

In the present formulation, optimal spreading is only achieved with very narrow channels, but it was also shown that tuning the ionic strength of the buffer, which modulates

DNA persistence length through enhanced electrostatic repulsion in low salt (Baumann et al., 1997), enhances the degree of elongation ((Riesner et al., 2007), Fig. 16).

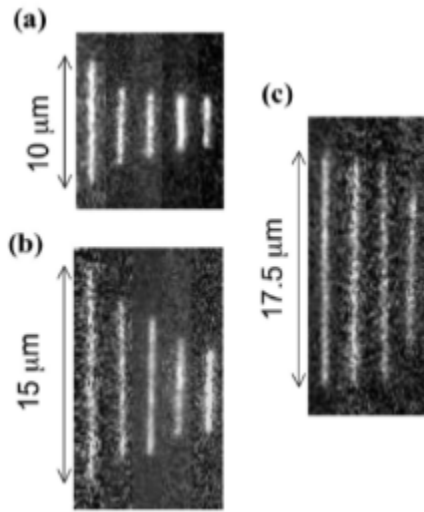


Figure 16: Fluorescence micrographs of λ -DNA molecules placed in (a) 200 nm, (b) 100 nm, and (c) 50 nm square nanochannels at different ionic strength (from left to right, 0.05x, 0.2x, 0.5x, 2x, 5x TBE; reprinted from (Riesner et al., 2007)).

Convinced by the potential of this technology, we launched this new activity at LAAS in 2008. We have now reached the expertise for high-throughput manipulation of batches of DNA molecules based on our own protocols for nanochannels fabrication (Viero et al., 2012). We note that this expertise is uncommon in biophysics mostly because it requires to be trained in cutting-edge nanofabrication facilities (Whitesides, 2011).

We recently investigated the response of single DNA molecules in nanochannels in the presence of hydrodynamic flow fields, whereas most nanofluidics experiments are carried out with electrophoresis, which is an efficient actuation strategy due to its facile implementation at the nanoscale⁶. Hydrodynamic flows are characterized by a Poiseuille field, in which the shear is maximal at the channels' walls and null at the centerline. Considering a fluid moving at 100 $\mu\text{m/s}$ and a 200 nm square nanochannel, the shear rate is $\sim 1000 \text{ s}^{-1}$, inducing a sufficient stress to extend single DNA molecules in bulk (Smith et al., 1999). We thus measured the length of single DNA molecules in the time course of their migration in nanochannels with hydrodynamics or electrophoresis, and we observed that the degree of elongation of a λ -DNA molecule was enhanced with hydrodynamic flow fields (Fig. 17A). This original spreading method has been patented because it enables to control the conformation of the DNA with the same geometry of nanochannels, and without using uncommon salt concentration, which allows to precisely monitor the conformation of DNA inside nanochannels (Fig. 17B). We are now working on the optimization of protocols to manipulate long chromosome fragments of several Mbp in length (not shown).

⁶ The reduced size of nanochannels is accompanied by a dramatic increase in hydrodynamic resistance (see e.g. Abgrall, P., Bancaud, A., and Joseph, P. (2010). Nanofluidic devices and their potential applications. In *Microfluidic Devices in Nanotechnology Fundamental concepts*, Wiley, ed., pp. 155-214.), meaning that very high pressures are required to convey DNA molecules in nanochannels, and implying the use of specific tubings with high resistance to pressure.

A

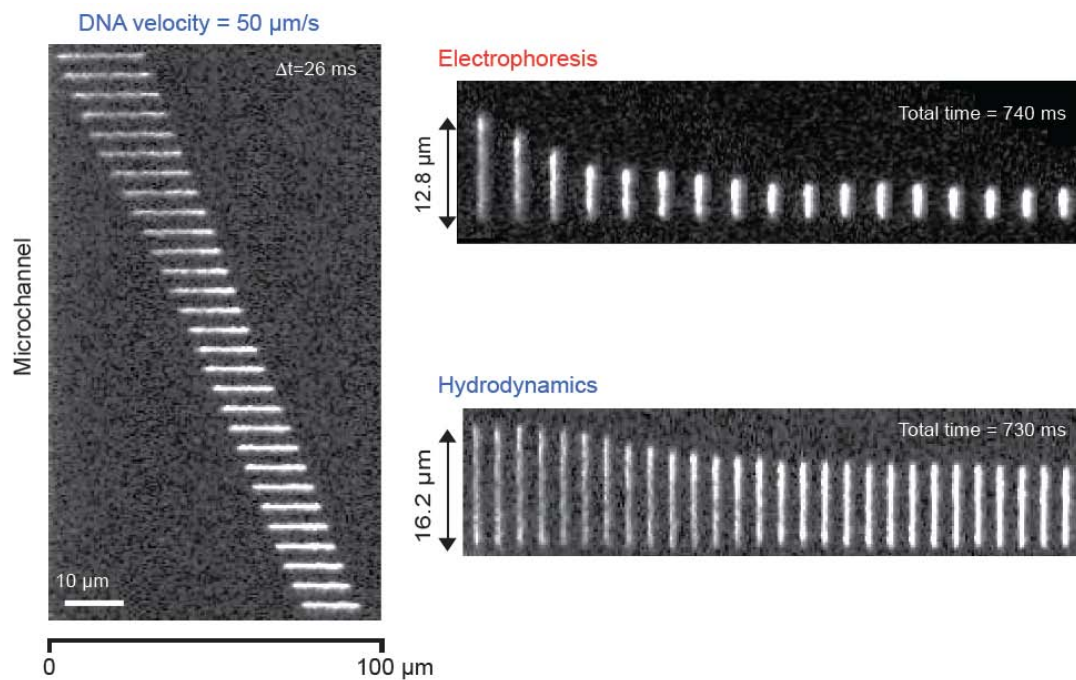
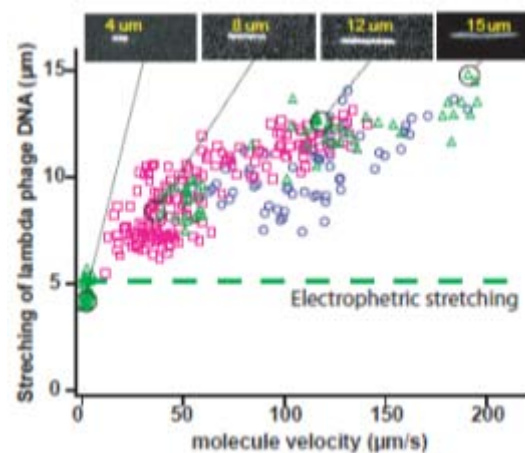


Figure 17 : **(A)** The left panel shows a time series of a λ -DNA in the course of its migration inside one nanochannel. The DNA velocity is 50 $\mu\text{m/s}$, and the time interval between two consecutive images is 26 ms. The right panel represents two molecules after the registration of their lagging edge using electrophoretic and hydrodynamic actuation. The elongation is enhanced in the case of hydrodynamics. **(B)** The degree of elongation can be tuned, as shown by the increase in extension with the velocity of the molecule inside nanochannels (dots in the right panel). This effect is not observed with electrophoresis (dashed line in the right panel). This spreading method driven by hydrodynamics has been patented.

B



DNA methylation: storing epigenetic information at the DNA level

DNA methylation is one of the best-studied epigenetic modification of mammalian genomes. It predominantly occurs at CpG dinucleotides, which are reversibly methylated at the 5' position of cytosine by specific enzymes called DNA methyltransferases (DNMT, Fig. 18). CpG dinucleotides are sparsely distributed through the genome except at **short genomic regions called CpG islands (CGI)**. A CpG island is defined as a sequence with a GC content that is greater than 55% and ratio of CpG to GpC of at least 0.65 (Takai and Jones, 2002). CpG islands are at least 500 base pairs long, and they are mostly found in the proximal of promoter regions of almost half of the genes in the mammalian genome. Furthermore, different CpG sites are methylated in different tissues, resulting in a pattern of methylation

that is gene and tissue specific. This unique pattern of methylation confers upon the genome specific cell-type identity, and thus plays a central role in cellular differentiation and development.

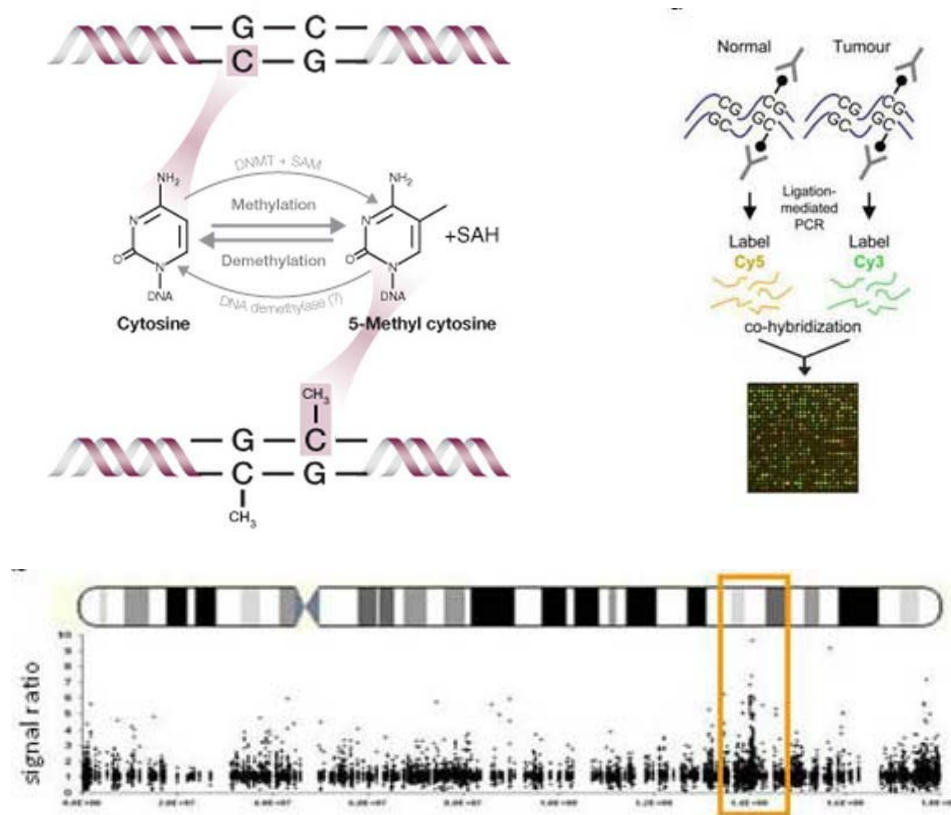


Figure 18 : The upper left panel shows the principle of methylation of DNA at the 5-carbon position of cytosine. This modification is performed by DNA methyl-transferases. The upper right panel depicts the analysis of methylated DNA from normal and tumor cells by Methyl-DNA immunoprecipitation (MeDIP), followed by microarray analysis at the genome-wide level. The lower image is the averaged MeDIP profile of a Wilms' tumor, showing a peak of DNA methylation on the long arm of chromosome 5 (reprinted from K. Malik group, Bristol).

CGI are, generally, unmethylated in normal cells, and **hypermethylation of promoter regions is the most well-categorized epigenetic change to occur in tumors**, particularly for a growing list of tumor-suppressor genes that are silenced by promoter hypermethylation. Methylation of gene promoters interferes with the binding of certain transcription factors, attracting DNA-binding proteins that in turn recruit other modifying enzymes leading to a chromatin configuration that is unfavorable to gene expression. Thus, DNA methylation is an effective means by which gene expression is silenced. In normal cells, DNA methylation functions to prevent the expression of imprinted and inactive X chromosome genes. In cancerous cells, DNA methylation inactivates tumor-suppressor genes, as well as DNA repair genes, can disrupt cell-cycle regulation, and activates (via hypomethylation) certain oncogenes. Many studies have thus recently investigated the aberrant patterns of DNA methylation in cancers using methylated DNA immuno-precipitation (MeDIP, Fig. 18), a

technology very similar to conventional ChIP that can be brought to the genome-wide level in combination with e.g. micro-array analysis (see section 3.b). DNA methylation patterns now seem to be of value as cancer markers for tumor prognosis, and predictors to chemotherapy (Mulero-Navarro and Esteller, 2008). Interestingly the reversible nature of epigenetic changes is generally viewed as appealing for future “reversible” chemotherapies.

This growing field of research has also attracted the attention of biophysicists, and different assays were developed for methylation profiling. The following list of examples shows that proof-of-principle experiments have been carried out, but the efforts to bring these technologies at the genome-wide level remain to be performed. First DNA unzipping experiments were performed by AFM on methylated or unmethylated 20-bp fragments, showing that the adhesion energy of the two strands was altered in methylated DNA (Severin et al., 2011), and suggesting that mechanical identification of methylation could be carried out. In another direction many efforts have been carried out to detect CGI on chromosomes using DNA combing, and preliminary results have demonstrated that methylated regions could be identified on λ -DNA templates (Cerf et al., 2011). Finally methylated fragments were ligated to unmethylated DNA in order to produce barcodes that were convincingly characterized by light microscopy in nanochannels (Fang et al., 2011).

b- The nucleosome level

The nucleosome is the fundamental unit of chromosomes. We propose to overview its structure, its dynamics, and finally its response to mechanical constraints.

Crystal structure of the nucleosome core particle

The cylindrical shape of the nucleosome, which involves 147 \pm 2 DNA bp wrapped around four histones in the following order H2A, H2B, H3 and H4 (Fig. 19), was described at an overall resolution of \sim 20 Å in 1983 (Richmond et al., 1983). The first long-awaited atomic structure of the nucleosome was disclosed in 1997 by Luger and colleagues (Luger et al., 1997), using a DNA fragment that was a palindromic inverted repeat of a 73-bp, which greatly helped in growing diffracting crystals (Fig. 19). In addition to this peculiar sequence, the nucleosome core particle of Luger and colleagues was studied in highly unphysiological salt conditions, *i.e.* 75 mM MnCl₂, suggesting that atomic details of the particle should be analyzed cautiously.

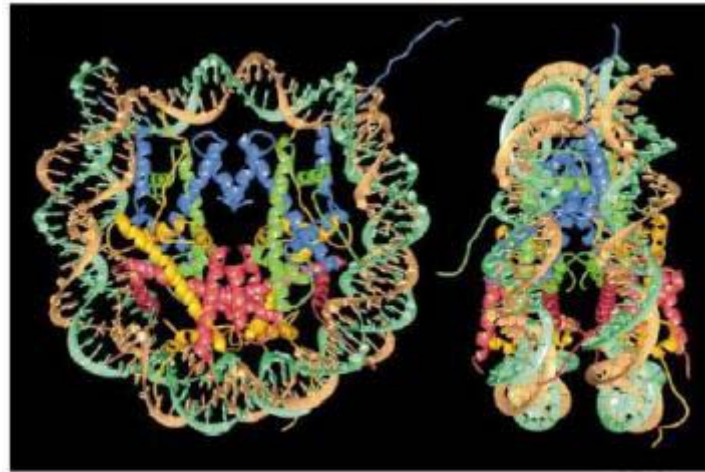


Figure 19 : Crystal structure of the nucleosome, showing DNA spooling around a protein core (reprinted from (Luger et al., 1997)).

Histones are positively charged proteins, which are highly evolutionary conserved. The DNA path around the histone octamer encounters one H2A/H2B heterodimer, then one (H3/H4)₂ heterotetramer, and another H2A/H2B heterodimer. Note that the (H3/H4)₂ heterotetramer complexed with DNA is called the tetrasome. DNA wrapping around the nucleosome corresponds to 1.65 turn of left-handed super-helix, inducing a negative writhe of 1.65 turn on DNA. DNA is sharply bent around the octamer, the mean curvature radius around the octamer being 4.2 nm (Prunell, 1998), and the nucleosome structure is stabilized by electrostatic interactions with regularly distributed every ~10 bp electrostatic DNA-protein attachment points. Histones share common structural characteristics, which are associated to a central globular domain, and long unstructured N-terminal, and to a lesser extent C-terminal, highly positively charged tails. Histone tails amount for 25-30% of the mass of core histones, and they can be subjected to a wide variety of posttranslational modifications, including acetylation and methylation for lysine residues, phosphorylation for serines or ubiquitylation (Fig. 20), that confer to histones functional roles in DNA transactions (see more below). Their conformation on nucleosome core particles is complex because they NMR studies demonstrated that tails are bound to nucleosomal DNA in low salt conditions, and they are released and mobile in close to physiological salt conditions ~0.3 M NaCl (Smith and Rill, 1989; Walker, 1984). Interestingly, despite their attractive roles for biochemical modifications, tail domains contribute marginally to the stability and conformation of nucleosome core particles (Ausio et al., 1989; Hayes et al., 1991). Finally, a growing body of histone variants has also been identified for histone H3 and H2A. The best studied example is CenpA, which is a centromeric variant of H3 that plays an essential role in chromosome segregation during mitosis (Palmer et al., 1987).

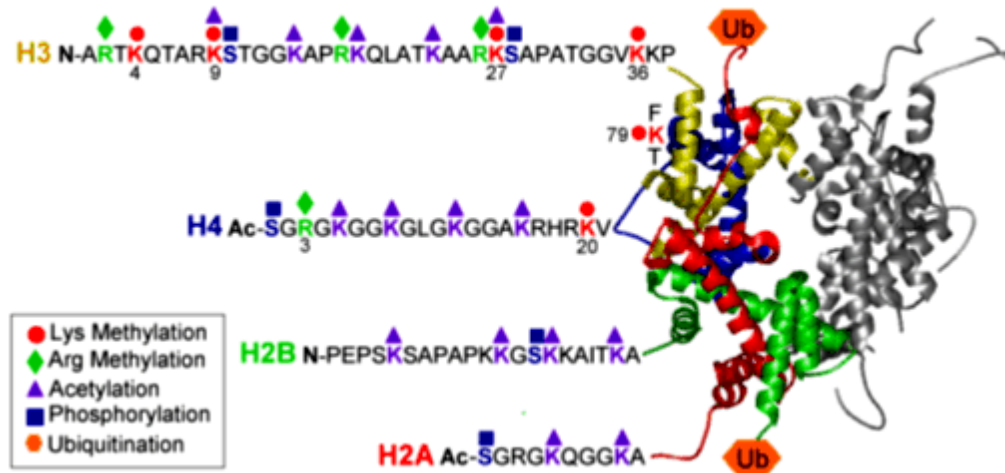


Figure 20 : Specific amino acid sites of posttranslational modifications (acetylation, phosphorylation, ubiquitination and methylation) that are known to occur on histones are indicated by colored symbols. Half of the structure of the nucleosome core particle H3 (yellow), H4 (blue), H2A (red) and H2B (green) are shown in color. The other half is represented in grey.

Taken together these biochemical features suggested that nucleosomes were versatile entities that played a central role in the regulation of biological transactions (Lavelle and Prunell, 2007). Interestingly though the commonly shared structural picture of the nucleosome is static, presumably due to the frozen view inferred from crystallography. The “tuna can” model, as ironically described by T. Owen-Hugues in 2004 (Flaus and Owen-hugues, 2004), is now known to be oversimplified.

Dynamics of the nucleosome entry-exit DNAs

Beyond the core particle, nucleosomes are characterized by entry-exit DNAs that were shown to be highly dynamic with the mini-circle approach (Prunell, 1998; Prunell et al., 1997). This technique consists in studying the behavior of mono-nucleosomes assembled on small DNA plasmids of ~350 bp given that the topology of these mini-circles can be finely analyzed by gel electrophoresis. This technique unraveled that the entry-exit DNAs in the nucleosome adopt three distinct conformations, namely open, negative and positive (Fig. 21). The negative conformation closely resembles the nucleosome core particle described by Luger with a wrapping of ~-1.7 turns around the nucleosome. The open conformation exists because entry-exit DNAs are strongly repulsive, leading to the dissociation of DNA-histone attachments at the edge of the nucleosome (Prunell and Sivolob, 2004). The open conformation is associated to a topology of ~-1 turn. In the positive state, which is associated to a topology of ~-0.5, entry-exit DNAs form a positive crossing, reducing the wrapping induced by the nucleosome (Sivolob et al., 1999). Although this conformation is stabilized by DNA-histone attachments at the edge of the nucleosome, its formation is less favorable than that of the negative and open states. Interestingly the discrepancy between the topology of crystallized nucleosome core particles vs. *in vivo* chromatin fibers, which are characterized by a topology of -1 turn per nucleosome lead to the linking number paradox, which remained unsolved for a long time. Prunell and collaborators proposed an

interpretation based on the spontaneous fluctuations of the entry-exit DNAs of the nucleosome to explain the paradox (Prunell and Sivolob, 2004).



Figure 21 : Representation of the three nucleosome conformations, as inferred from the minicircle approach.

The three nucleosome states coexist in a thermodynamical equilibrium, their difference in energy being on the order of thermal fluctuation energy (kT). In addition, their statistical distribution is finely regulated by a number of physical and bio-chemical parameters, including ionic conditions (Sivolob et al., 1999), the DNA sequence (Sivolob et al., 2003), histone-tail post-translational status (De Lucia et al., 1999), or the presence of histone variants e.g. CenpA (Conde e Silva et al., 2007). Interestingly, these results on the conformational flexibility of mono-nucleosomes were recently confirmed by an independent method based on bulk-FRET measurements (Gansen et al., 2009). Overall, nucleosomes appear as polymorphic entities, which can be regulated by physical as well as bio-chemical mechanisms.

The minicircle approach was also used to investigate the rotational dynamics of chromatosomes (that is, nucleosomes with linker histones), showing that linker histones form a stem motif, locking the entry-exit DNAs in the positive and negative configurations that were detected for nucleosomes (Sivolob and Prunell, 2003), and these two states are in dynamic equilibrium.

Multi-step tension-mediated unwrapping of single nucleosomes

The effect of tension on the stability of nucleosomes was first probed by optical tweezers, which demonstrated that nucleosomes could be disrupted when exerting forces larger than ~ 25 pN (Cui and Bustamante, 2000). The disruption mechanism was then precisely characterized using nucleosome arrays reconstituted by salt dialysis, showing a multistep eviction (Brower-Toland et al., 2002). At low stretching force 76 bp of DNA are released from the histone core, and 80 bp are subsequently released at higher forces. Interestingly when arrays were relaxed before complete dissociation of nucleosomes, they were able to re-assemble, showing the reversible nature of nucleosome disassembly.

In addition to the rapid unfolding of the entry-exit DNAs that is consistent with the results of Prunell and collaborators (see above), Wang suggested that the step-wise ejection of nucleosomes was associated to higher energies between DNA and the octamer at the dyad (red regions in Fig. 22A). A quantitative interpretation was then proposed (Kulic and Schiessel, 2004), showing that the geometry of the nucleosome is associated to an energy barrier after the eviction of 80 bp (Fig. 22B-C).

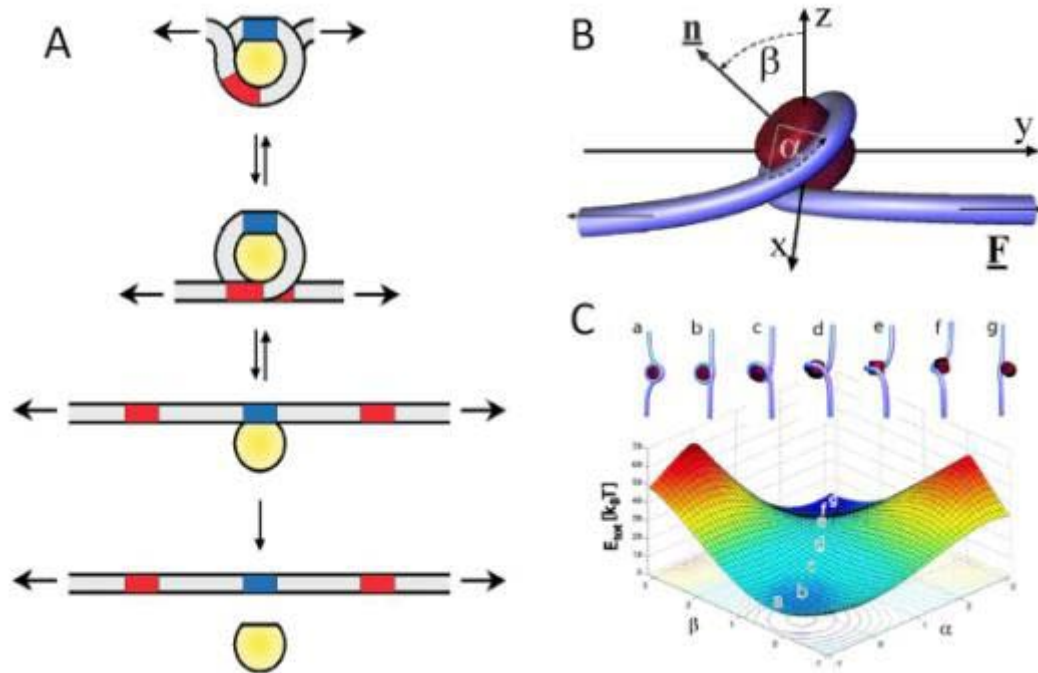


Figure 22: (A) Qualitative model accounting for the step-wise ejection of nucleosome when applying tension to a nucleosome array (reprinted from (Brower-Toland et al., 2002)). **(B-C)** The existence of a two-step disruption is related to the geometry of nucleosomes according to Schiessel's model, which shows the existence of a geometrical energy barrier associated to the spooling of DNA around the octamer (Kulic and Schiessel, 2004).

Single molecule studies of the stability of single nucleosomes were further refined on single nucleosome arrays (Hall et al., 2009) by mechanically unzipping the DNA wrapped around the octamer. The authors provided a detailed map of histone-DNA interactions along the DNA sequence to near base pair accuracy, revealing a distinct 5-bp periodicity that was enveloped by three regions of strong interactions, with the strongest occurring at the dyad. Unzipping up to the dyad allowed recovery of a canonical nucleosome upon relaxation, whereas unzipping beyond the dyad disrupted the octamer. Consequently, these studies provide a unique view on the interaction patterns governing the energies governing DNA-protein interactions at the nucleosome level with potential implications for RNA polymerase processing through nucleosomes.

The reversome hypothesis: when nucleosomes accommodate positive supercoiling

Magnetic tweezers have also been used to characterize the rotational response of nucleosome arrays assembled on tandem repeats of the 5S nucleosome positioning sequence. These experiments revealed an unexpected hysteretic behavior upon application of large positive stress to chromatin fibers, which could transiently accommodate the constraint by trapping positive turns at a rate of one turn per nucleosome ((Bancaud et al., 2007), Fig. 23A). Based on a comparison with the response of fibers of tetrasomes (the [H3-H4]₂ tetramer bound with ~50 bp of DNA), it was suggested that the trapping reflects a nucleosome chiral transition to a metastable form built on the right-handed tetrasome (Hamiche et al., 1996). This scenario was then tested by molecular modeling, which

suggested a mechanism for this structural transition and rather validated model II in Fig. 23B (Lavelle et al., 2009). This new nucleosome structure was coined the reversome.

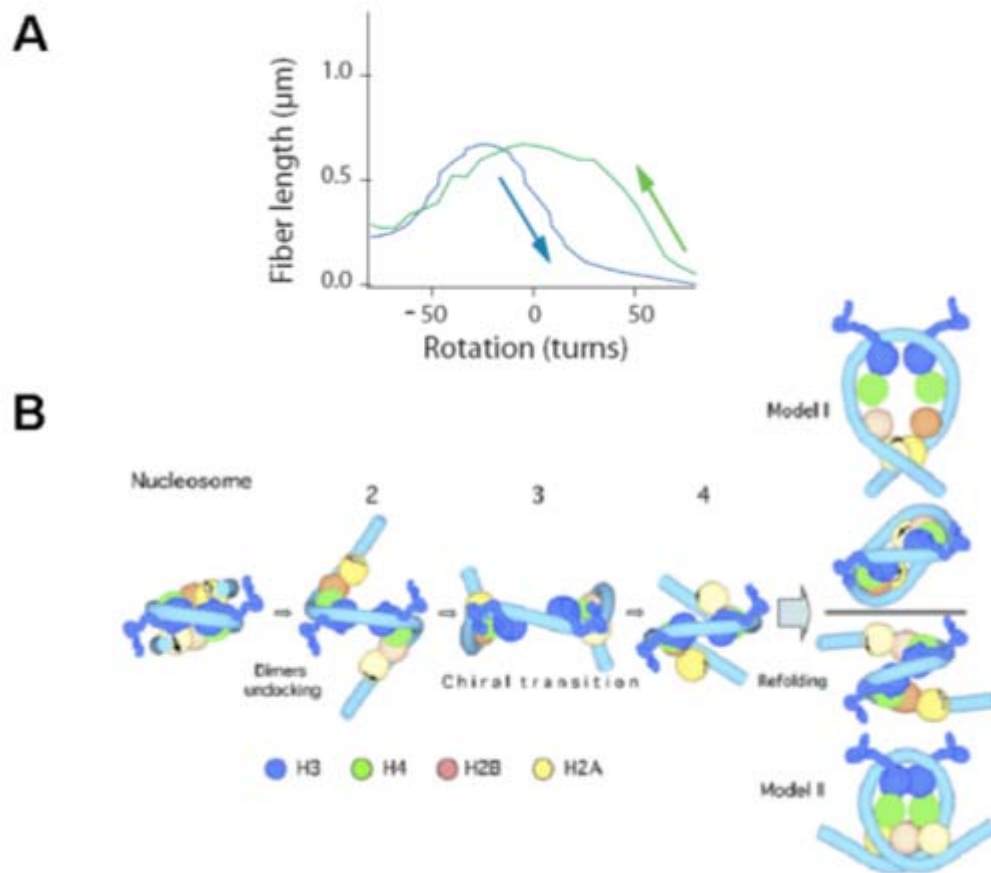


Figure 23 : (A) Plot of the fiber length vs. rotation, as measured with a magnetic tweezers set-up. Upon application of large positive torsion (blue forward curve), the fiber exhibit an hysteretic response when releasing the constraint (green backward curve), which is associated to the trapping of one positive turn per nucleosome. (B) Molecular scenario for the structural transition occurring at the nucleosome level. Molecular dynamic simulations tend to support model II (Lavelle et al., 2009).

Now, one may speculate on the relevance of this unstable nucleosome structure. In view of its low energy, <8 kT, it was initially proposed that this transition served to break the docking of the dimers on the tetramer that in the absence of other factors exerts a strong block against elongation of transcription by the main RNA polymerase. An unexpected result also supported the relevance of this altered nucleosome structure: Henikoff and colleagues characterized the topological structure of CenpA containing nucleosomes and observed that these particles stored one positive turn (Furuyama, 2009), as in the reversome. Though these authors did not mention our work most likely intentionally (Lavelle et al., 2009), they provided strong support to the reversome hypothesis. Finally, it is always questionable whether the mechanical response of nucleosome arrays depleted from linker histones (H1/5), which are abundant in mammalian cells and dynamically attached to the entry-exit of the nucleosome, is relevant to *in vivo* molecular transactions. Recouvreux *et al.* thus set out to investigate the torsional response of chromatin fibers comprising linker histones (Recouvreux et al., 2011). They showed that chromatosomes could undergo a reversible

chiral transition toward a state of positive torsion (reverse chromatosome) without loss of linker histones.

c- The nucleosome array level

Nucleosomes are regularly repeated *in vivo*, and the repeat lengths spans from 165 bp to 260 bp in yeast and sea urchin, respectively (van Holde, 1989). This large scale organization has been proposed to direct the folding of nucleosome arrays into a 30 nm fiber. In this section, we first describe the mechanisms of compaction that were observed *in vitro*, and we briefly review the models of the 30 nm fiber. We then overview the literature on the mechanics of nucleosome arrays, and we finally raise open questions on epigenetic inheritance, *i.e.* how is chromatin epigenetic structure inherited through the cell cycles?

Folding of nucleosome arrays in a compact fiber

Electron microscopy (EM) was among the pioneering techniques to study chromatin structure, and it provided one of the most fundamental evidence for nucleosome existence based on the visualization of regular bead on a string structures (Oudet et al., 1975). These images were obtained after harsh purifications, and huge efforts were carried out to purify chromatin fibers under close to physiological ionic conditions (Fig. 24A). Salt conditions appeared to be critical to preserve the folding of chromatin fibers, because separated beads on a string structure were obtained in low salt conditions, whereas compact 30-nm fibers were observed with salt concentrations larger than 80 mM (Gerchman and Ramakrishnan, 1987). Linker histone H1 appeared as a critical factor to direct the folding of a compact structure because atomic force microscopy (AFM) measurements showed that fibers depleted of H5 could not fold into a compact state ((Leuba et al., 1998a; Leuba et al., 1998b), Fig. 24B). Other chromatin binding proteins are also sufficient to direct the folding into a compact state, as inferred from electron micrographs of polycomb-containing nucleosome arrays (Francis et al., 2004). Interestingly, solution conformation of native chromatin and fully defined chromatin arrays obtained by cryo-EM appeared to be consistent with a zig-zag model for the 30 nm fiber, which is characterized by straight linker DNAs crossing chromatin super-helix axis (Bednar et al., 1998). This model was supported by AFM measurements (Leuba et al., 1994), and the folding into a 30 nm fiber was also shown by X-ray diffraction performed on oriented chromatin fibers that were purified from chicken erythrocytes (Widom and Klug, 1985).

Studies on chromatin compaction in a compact state received renewed interest, as emerged the DNA sequence with optimal affinity for nucleosomes, coined the Selex 601 sequence (Lowary and Widom, 1998), because nucleosome arrays could be prepared with acute control over the distribution of nucleosomes along the array. Compact nucleosome arrays with diameters strongly dependent on the nucleosome repeat length were then observed by electron microscopy (Robinson et al., 2006), and this result appeared to be in contradiction with the zig-zag model. However, the tetra-nucleosome crystal structure of the same sequence with a repeat length of 160 bp supported the zig-zag model (Schalch and

Duda, 2005). Altogether these results emphasize the difficulty in properly resolving the structure of the 30 nm fiber, although the propensity of chromatin to fold into a compact fiber is clearly evidenced by imaging techniques.

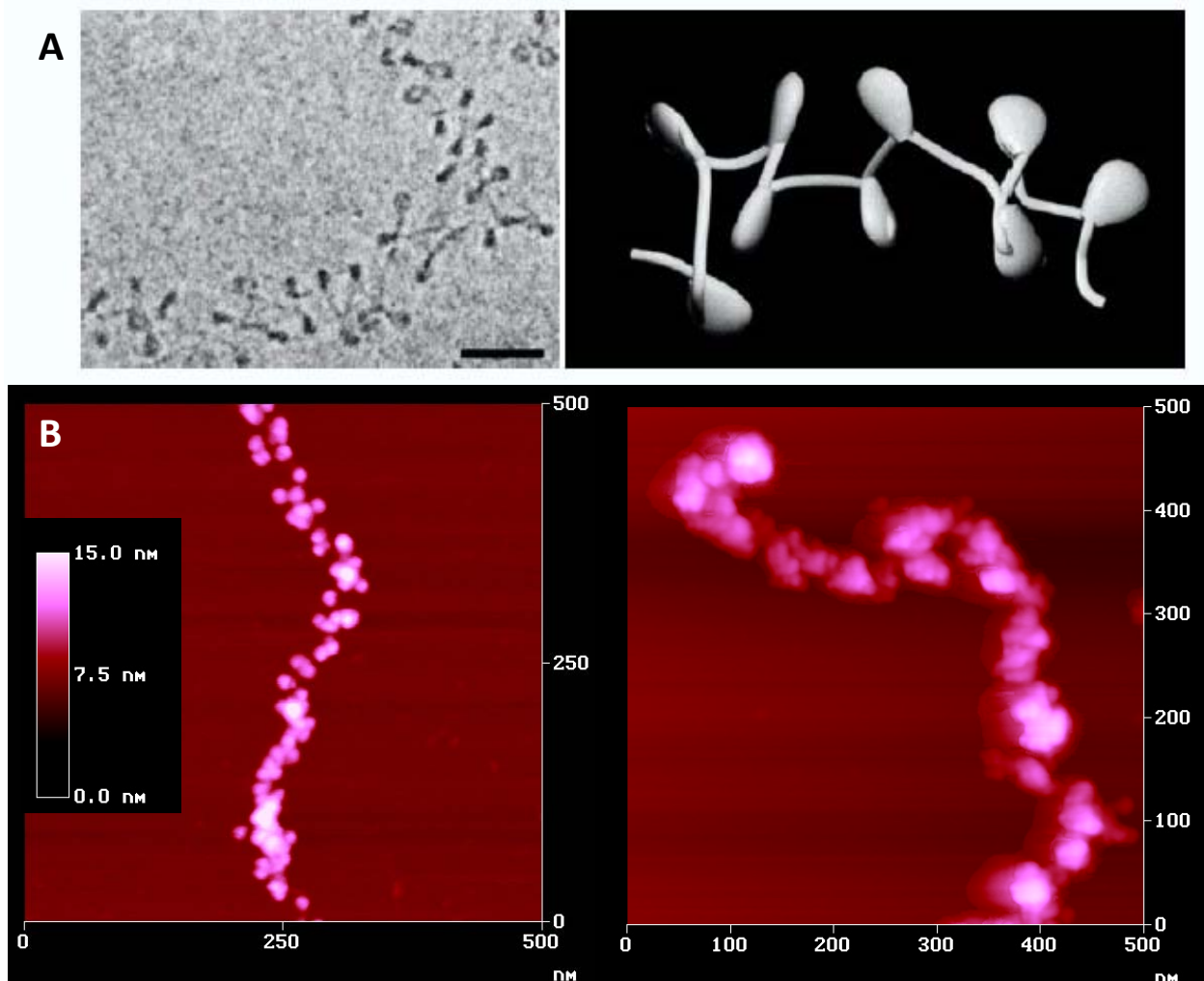


Figure 24: (A) Native chromatin fibers observed by transverse electron microscopy in thin cryo-section at 5 mM NaCl, and the corresponding model of nucleosome with stem motifs (Bednar et al., 1998). (B) Similar native chromatin fibers observed by atomic force microscopy at 10 mM NaCl and 60 mM NaCl (left and right pictures, (Leuba et al., 1998c)).

Interestingly analytical ultracentrifugation has also been used to assay the folding of chromatin. This technique consists in measuring the sedimentation coefficient of nucleosome arrays (in Svedberg (S)⁷). The behavior of chromatin fragments containing 12 nucleosomes assembled on positioning sequences was studied in various salt conditions, and the sedimentation coefficient appeared to increase from 29 S in low salt conditions to a maximum of 55 S in the presence of divalent cations (Schwarz and Hansen, 1994), this value being consistent with the formation of a fiber of 30 nm in diameter (Hansen, 2002b). Note that similar results, namely the increase of the hydrodynamic radius with salt concentration, were obtained by gel electrophoresis (Fletcher et al., 1994). Interestingly, linker histones

⁷ The Svedberg is the ratio of the molecular weight to the viscous drag.

appeared to accelerate the folding into a compact nucleosome arrays because lower concentrations of divalent cations were sufficient to reach the 55 S conformation.

Histone tails are known to be finely regulated by post-translational modifications, and their functional role for chromatin folding has been extensively studied. First, tailless histones were shown to be deficient to direct the folding in a 30 nm fiber even in the presence of monovalent and divalent salt (Carruthers et al., 1998). The role of individual tails was also assessed, and the deletion of H3 N-terminal tail was found to be essential for the proper folding of long chromatin fragments (Leuba et al., 1998a), most likely because nucleosomes preferentially adopt the open state that is not optimal to reach a compact conformation. The same observation was carried out with H4 N-terminal tail deleted nucleosomes (Dorigo et al., 2003), but the mechanism, which seems to involve long range nucleosome-nucleosome interactions, is different. The role of post-translational modifications was also investigated using hyper-acetylated histones, which bear a reduced net charge. Chromatin fibers exhibited a bead on a string structure in electron micrographs even in physiological salt conditions (Garcia-Ramirez et al., 1995). The most recent studies demonstrated that single post-translational modifications could also play a key role for chromatin higher order organization because engineered H4 bearing one post-translational modification remained uncondensed (Shogren-Knaak et al., 2006). Finally the role of histone variants in chromatin higher order organization was investigated, and CenpA-containing fibers were shown to remain extended even in physiological salt conditions (Dalal et al., 2007). Interestingly the deficit of CenpA fibers to fold in a compact fiber may be linked to the preferential formation of open nucleosomes with this variant, as inferred from the minicircle approach (Conde e Silva et al., 2007).

Structure of the 30 nm fiber: chasing a mirage!

The historical structural model of chromatin is the solenoid model proposed by Finch and Klug in 1976 (Finch and Klug, 1976). This model assumes that nucleosomes are stacked on each other, and located sequentially around the fiber. It has never been demonstrated convincingly in part because its suggestion relied on electron micrographs, in which the DNA path inside the fiber could not be resolved. In addition this model implies that linker DNAs joining consecutive nucleosomes are strongly bent. This energetic cost has to be balanced by strong nucleosome-nucleosome attractive forces, but there is no clear consensus on this stacking energy.

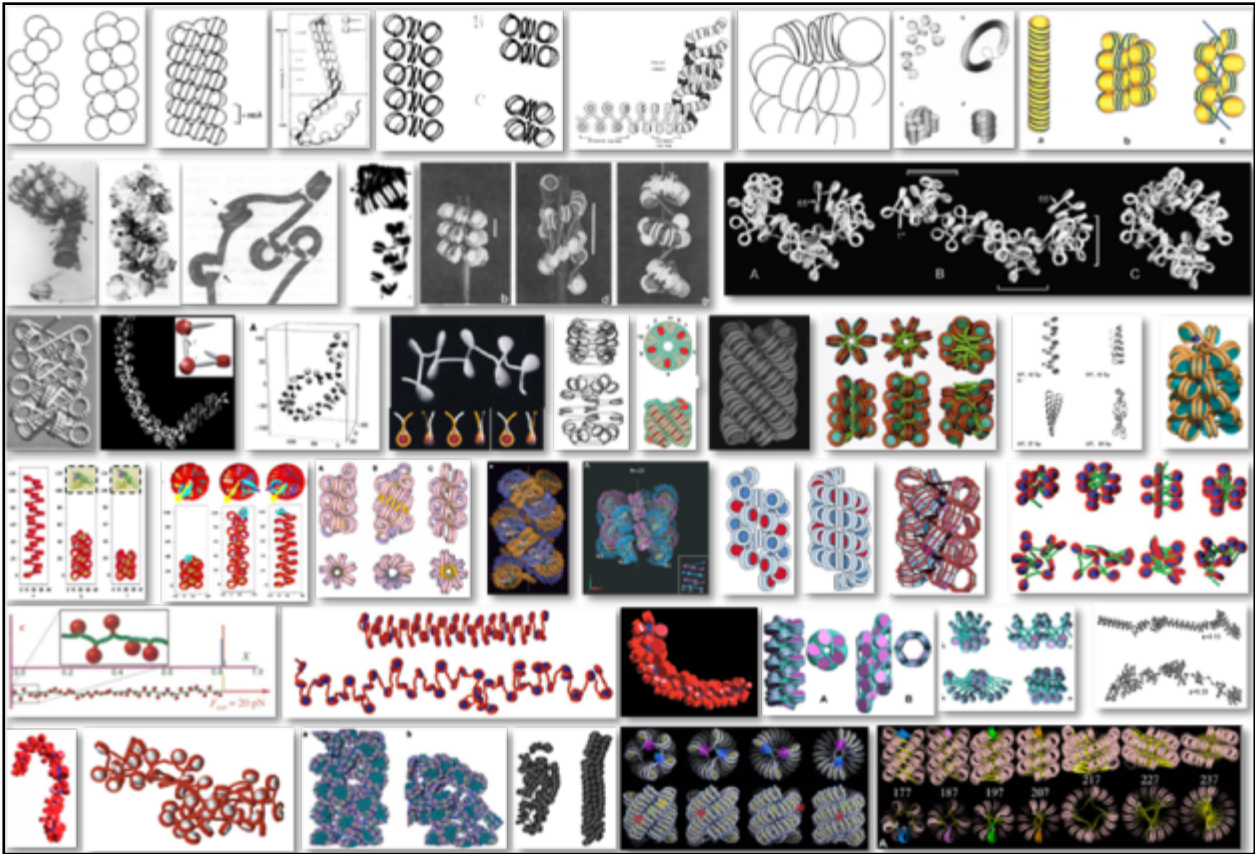


Figure 25 : We picked up a picture proposed by Mozziconacci and Lavelle (Mozziconacci and Lavelle, 2009), showing a collection of chromatin fiber models gathered from the abundant literature on this subject. These models include schematic drawings, hand-made models, computer-assisted geometrical and/or computational models.

The zig-zag model was proposed in 1993 (Woodcock et al., 1993), and it quickly gained popularity in the community. It postulates straight linker DNAs that cross the super helix axis, and involves three geometrical parameters, namely the DNA linker length, the DNA entry-exit angle, and the relative angle between consecutive nucleosomes. Interestingly, this model predicts that chromatin diameter seldom depends on nucleosome repeat length, and this proposition was recently invalidated by cryo-EM obtained on tandem repeats of the 601 sequence assembled with linker histone. This technique indeed showed that arrays with repeat lengths spanning 177 to 207 bp fold into a fiber of ~ 35 nm in diameter and 11 nucleosomes/11 nm in compaction, while longer arrays produce fibers of 45 nm in diameter and 15 nucleosomes/11 nm (Robinson et al., 2006).

The controversy between the zig-zag and the solenoid models thus remains unsolved, and the analysis of the extensional response of single chromatin fibers was equally well reproduced with the zig-zag (Ben-Haïm et al., 2001; Katritch et al., 2000; Schiessel et al., 2001; Wedemann and Langowski, 2002), and the solenoid model (Kruithof et al., 2009). Notably it was recently suggested that chromatin fibers could adopt several conformations, including solenoids, 2-start helix, 3-start helix, and 5-start helix using inverse kinematics and docking algorithms to place linker histones (Wong et al., 2007).

Furthermore, it must be pointed out that 30 nm fibers were never convincingly detected *in vivo* using high resolution electron microscopy of thin nuclear sections, which rather pointed to the existence of fibers with a variety of diameters, none particularly resonant with a hierarchical organization built on a 30 nm structural element (Woodcock and Ghosh, 2010). Overall, chromatin higher order structure at the 30 nm fiber level remains unelucidated, despite considerable experimental and modeling efforts for ~40 years (Fig. 25).

Mechanics of single nucleosome arrays

The extensional response of nucleosome arrays was examined with single molecule techniques in the low force regime (that is, before individual nucleosome start to be disrupted by the force), and this response was generally analyzed with the worm-like chain to extract the persistence length of chromatin fibers. These studies were conducted on nucleosome arrays (Bancaud et al., 2006b) or chromatosome arrays (Kruithof et al., 2009) obtained by salt dialysis, on chromatin assembled with *Xenopus Laevis* eggs extracts (Bennink et al., 2001), or on chromatin fibers purified from chicken erythrocytes (Cui and Bustamante, 2000), consistently showing that chromatin persistence length is ~20-30 nm. This low value for the persistence length of chromatin in comparison to naked DNA testifies for the fact that chromatin is a stiffer spring than DNA in the low force regime. Interestingly *in vivo* measurements of chromatin persistence length of ~200 nm by light microscopy techniques (Bystricky et al., 2004b) and by chromosome conformation capture techniques (Dekker, 2008) depart from these *in vitro* data, and this contradiction still needs to be addressed experimentally (see more in section on chromosome movements in living cells).

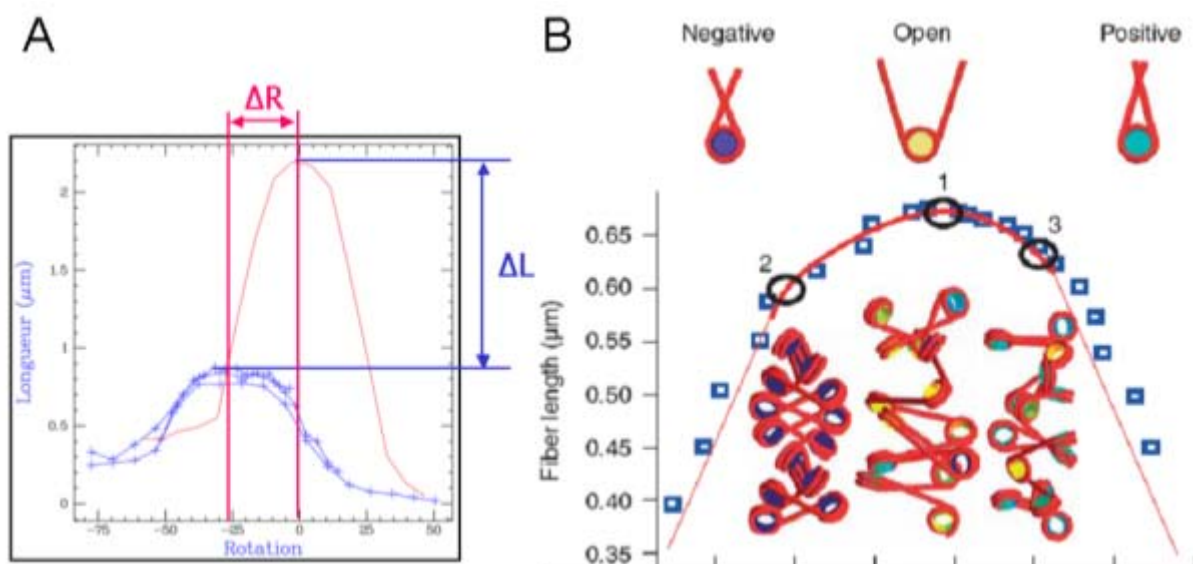


Figure 26: (A) The force applied to the fiber is maintained at 0.2 pN, and the length of the molecule is monitored for different topological constraints. The response of chromatin (blue) is compared to that of DNA (red), as measured after complete dissociation of the nucleosomes using hepatin rinsing. **(B)** Chromatin response to torsional constraints is modeled by a three-state model of the nucleosome, as represented in the

upper part of the graph. The molecule accommodates large topological deformation without change in length to the dynamic equilibrium between these three nucleosome states.

We devised a magnetic tweezers set-up to investigate the rotational behavior of nucleosome arrays (Bancaud et al., 2006a) and of chromosome arrays (Recouvreux et al., 2011). These studies disclosed the torsional resilience of chromatin fibers, which is characterized by an exceptionally low rotational persistence length of ~ 5 nm in comparison to naked DNA (80 nm, Fig. 26A). This signature was explained quantitatively by setting up an analytical model of torsion comprising the existence of three nucleosome conformations in thermodynamic equilibrium (see Fig. 26B). This model suggested that the rotational flexibility of nucleosomes could serve as a topological buffer capable of accommodating large constraints. This model also implies that the chromatin structure is engineered at the molecular level to accommodate torsional constraints occurring e.g. upon DNA elongation by polymerases.

Assembly of nucleosome arrays

As for the architecture of chromatin, several questions on the assembly reaction of this structure remain unanswered. Early contributions elucidated that nucleosome assembly was a three-step mechanism associated to (i) $(H3-H4)_2$ heterotetramer assembly, and (ii) the deposition of two $(H2A-H2B)$ dimers consecutively. This result was first established in 1978 using *Drosophila melanogaster* tissue culture cells (Worcel et al., 1978) and by radio-labeling newly incorporated histones. It was also detected *in vitro* during salt-dialysis assembly reactions, as well as under physiological conditions (Ruiz-Carrillo et al., 1979; Wilhelm et al., 1978).

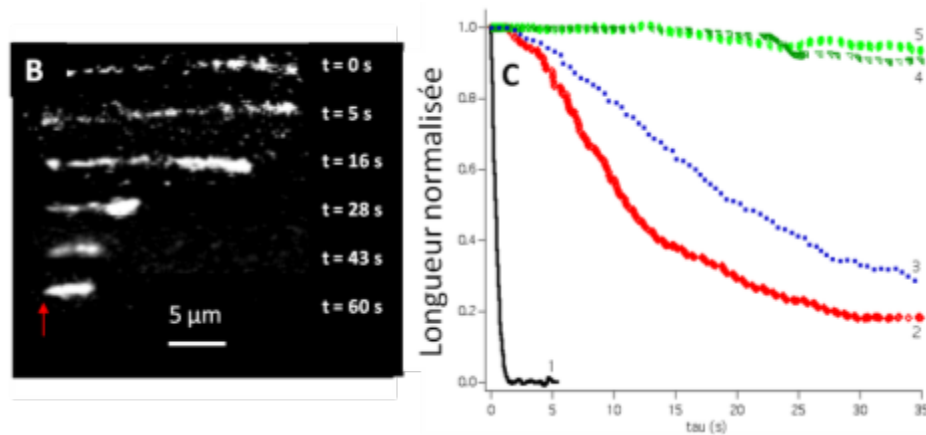


Figure 27 : (B) Time series of a single DNA molecules over time as a solution of purified histones is flowed in the fluidic device. The shortening of the molecule accounts for the formation of chromatin. The red arrow indicates the anchoring point of the molecule. (C) The plot shows the normalized length over time as molecules are incubated with different chromatin assembly systems using a histone concentration of 1,5 ng/μL. (1- xenopus eggs extracts, 2-native histones + NAP-1, 3-native histones, 4- native histones + poly-glutamic acid, 5- native histones + RNA). Note that the reaction with purified reconstitution systems follow a slow and sequential mechanism, compatible with the deposition of one $(H3-H4)_2$ tetramer followed by two $(H2A-H2B)$ dimers. Faster compaction kinetics and higher packing ratios are reproducibly reached with egg extracts.

The kinetic parameters governing this reaction have remained poorly characterized for a long time because conventional molecular techniques could not precisely assay dynamic reactions. We used our technology to elongate single DNA molecules in shear flow (described in section 4a) to characterize the chromatin assembly reaction in real time with different biological systems: *Xenopus Laevis* egg extracts, purified nucleosome reconstitution systems using a combination of histones with either the histone chaperone Nucleosome Assembly Protein (NAP-1) or negatively charged macromolecules such as polyglutamic acid (PGA) and RNA (Fig. 27C, (Wagner et al., 2005)). The comparison shows that the compaction rates can differ by a factor of up to 1000 for the same amount of histones. The faster reaction rate is detected with egg extracts (black dataset). The formation of nucleosomes is accelerated in the presence of NAP-1 (red dataset compared to the blue one), but dramatically slowed down with the unphysiological histone chaperones RNA or PGA (green datasets). Magnetic tweezers were also used to study the chromatin assembly reaction (Leuba et al., 2003), allowing for an exquisite control on the force acting on the molecules. This study showed the strong dependence of the reaction rate with the force, and the inhibition of the reaction for forces exceeding 10 pN.

Notably the assembly systems described above (except for egg extracts) lead to the formation of fibers with randomly distributed nucleosomes, which cannot fold in a compact state. The identification of the protein complexes responsible for the assembly of regular nucleosome arrays has been a mainstream research direction in the 90's. The two most well-characterized families of chromatin remodeling factors are the SWI/SNF family (Muchardt and Yaniv, 1999) and the ISWI family (Langst and Becker, 2004). Their mechanism of action has been extensively studied by molecular biology techniques, showing nucleosome mobility is induced by ATP-dependent twist defect diffusion or bulge diffusion mechanisms (Flaus and Owen-Hughes, 2003). In addition, the real time dynamics of remodeling factors processing was investigated in a few single molecule studies. First, the action of one RSC complex (SWI/SNF family) on single DNA molecule was characterized by magnetic tweezers, showing the transient formation of negative supercoiling (Lia et al., 2006). This study also provided the first observation of DNA translocation by a remodeler at 200 bp/s over ~500 bp. The same experiment carried out on single nucleosome with optical tweezers also indicated that loops of DNA were formed on the nucleosome surface (Zhang et al., 2006), though the translocation properties of the remodeling complex seemed very different from those measured with magnetic tweezers (see (Lavelle et al., 2011) for discussion). The effect DNA translocation by remodeling factors on nucleosome positioning was directly scanned at the single molecule level by Wang and colleagues using a sophisticated DNA unzipping technique (Shundrovsky et al., 2006), which allows to determine the nucleosome position with an accuracy of 3 bp, and to detect (H2A-H2B) release. The processing of SWI/SNF induced a broadly-distributed movement of the nucleosome of 28 bp in average, which is much smaller than DNA translocation. Though more work is needed to help resolve this complex picture, it seems that the "inch-worm" model involving twist and loop propagation is most relevant to account for the data (Cairns, 2007).

Beyond the mechanism of nucleosome deposition and remodeling, many examples of epigenetic inheritance have been described, and, given that nucleosomes contain some of the main epigenetic imprints, many questions about the mechanisms that govern epigenetic inheritance are at the heart of modern biology (Fig. 28A). Let us first consider the example of transcriptional memory in yeast, which showed that gene-expression patterns could be durably altered, typically five generations (Ekwall et al., 1997). Similarly epigenetic memory has been detected for the genes involved in the galactose metabolism of budding yeast, because the kinetics of GAL gene activation are dramatically different depending on prior exposure of the cells to galactose (Brickner et al., 2007; Kundu et al., 2007). Interestingly many mechanisms have been invoked to account for this data, including histone variant substitutions, gene relocalization, or nucleosome remodeling. Despite these controversies in the literature, these results point to an important characteristic of epigenetic inheritance, which is sufficiently stable for being inherited through multiple cell cycles, and also sufficiently dynamic to be erased after some time.

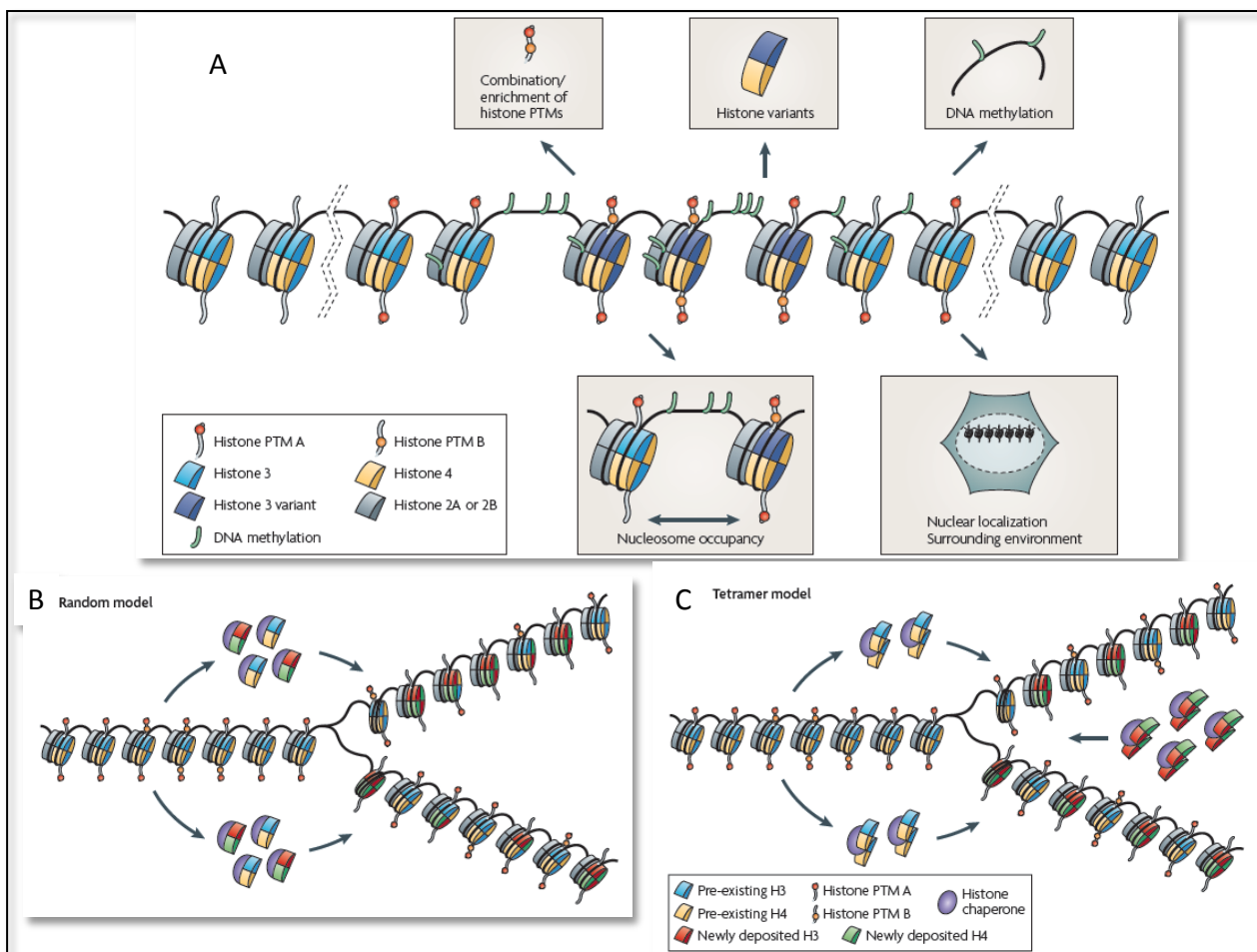


Figure 28 : (A) Schematic depicting physical and biochemical modifications that define different chromatin structures *in vivo*. The lower panel shows models of histone deposition during replication. (B) Random model of histone segregation, in which the histones segregate randomly between the leading and lagging replicated DNA strands. (C) Semi-conservative model of histone segregation, in which parental H3–H4 histones segregate

as tetramers, resulting in the joint deposition of recycled histones and newly deposited histones. PTM, post-translational modification (reprinted from (Margueron and Reinberg, 2010)).

In contrast to DNA replication, which is a semi-conservative process with one of the two DNA strands inherited by each daughter cell, the duplication of chromatin implies *de novo* histone deposition on newly synthesized chromatin fibers, as well as transfer of parental histones. In principle the transfer of parental histones could serve as a template on which to copy epigenetic information on newly synthesized histones. It is generally admitted that parental histones are disrupted into two (H2A-H2B) dimers and one (H3-H4)₂ tetramer, which may be reassembled either randomly or cooperatively on nascent strands with newly synthesized histones (Fig. 28B or C, respectively). The former model implies that histone modifications are diluted by the incorporation of new histones, and it is mostly considered as irrelevant.

The mechanisms governing a coordinated histone deposition remain to be fully understood, but it becomes increasingly clear that cells have evolved efficient chromatin-maturation mechanisms that reproduce chromatin organization in the wake of DNA replication (Groth et al., 2007; Margueron and Reinberg, 2010). We note that the protein PCNA (Proliferating Cell Nuclear Antigen), which is a ring-shaped trimeric protein that encircles DNA and serves as processivity factor for DNA polymerases (Fig. 29, (Kelman, 1997)), seems to play a key role for chromatin restoration. PCNA is retained on newly synthesized chromatin for ~20 minutes, meanwhile recruiting a large number of epigenetic-modulating factors, among which DNA methyltransferases (DNMT1), histone deacetylases (HDAC), chromatin remodeling factors (SNF) (Fig. 29). Notably, newly synthesized H3 and H4 are transiently acetylated at multiple lysine residues, and these post-translational mutations may serve as molecular signal to recruit specific factors involved in the maturation of *de novo* deposited nucleosomes.

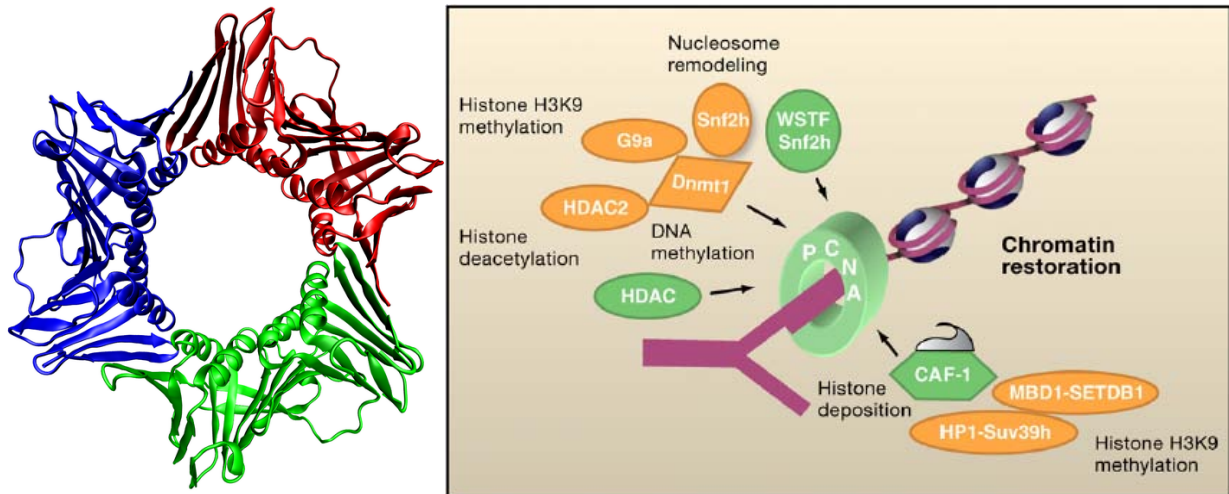


Figure 29 : The left panel shows the crystal structure of PCNA trimeric complex, and the right panel depicts known chromatin modulating factors interacting with PCNA and potentially involved in nucleosome epigenetic maturation after replication (reprinted from (Groth et al., 2007)).

d- Chromosome large scale organization and dynamics

We now focus on chromosome large scale organization. We first show that chromatin forms a continuous multi-scale structure in which large-scale patterns are characterized by epigenetic signals stored at the nucleosome level. We then review recent insights gained on how chromosomes fold, and we finally deal with spatio-temporal fluctuations in the nuclear context, either at the chromosome level or in the nucleoplasm.

Chromatin domains and epigenetic marks

The existence of large scale chromatin compartments was first detected in 1928 (Heitz, 1928) based on the observation of longitudinal differentiation of mitotic chromosomes. A bi-partite segmentation in heterochromatin and euchromatin was then proposed. Euchromatin is enriched in active genes and generally described as “accessible” (though this term is not well defined in the literature), whereas heterochromatin is highly condensed, poorly transcribed, and enriched in repetitive sequences. Heterochromatin controls several fundamental aspects of nuclear functions : (i) kinetochore assembly, (ii) sister chromatids cohesion (ensuring the proper segregation during cell division), (iii) repression of spurious recombination at repetitive sequences (maintaining genome integrity) (iv), repression of transcription of underlying and neighboring sequences, (iv) repression/activation of some long-range interactions involved in developmental regulation.

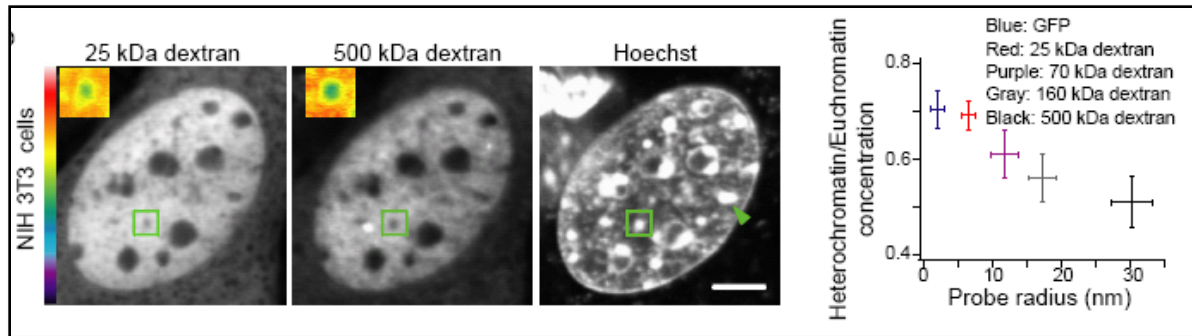


Figure 30 : The fluorescence micrographs represent the nucleus of an NIH3T3 cell micro-injected with a mixture of 25 kDa and 500 kDa dextran fluorescently labeled with two fluorophores. The chromatin density map is obtained using the DNA-intercalating agent Hoechst, and shows that inert tracers are excluded from heterochromatin foci, and this exclusion increases with the molecular weight of the probe (graph at the right). Insets are two-fold magnified zooms of the heterochromatin foci depicted by a green square. The scale bar represents 10 μ m.

At the molecular level, heterochromatin is distinguished by specific epigenetic hallmarks: hypo-acetylation of histones, methylation of lysine 9 of histone H3 (Peters et al., 2002), association with structural proteins of the HP1 family and strong periodic ordering of nucleosomes, which act in collaboration to produce a stable, compact and weakly accessible fiber. The distribution of these marks is now characterized at the genome wide level by ChIP techniques (see section 3c). Interestingly, although heterochromatin appears as a stable compartment by light microscopy during periods of hours, molecular interactions systematically appeared to be dynamic in heterochromatin (Cheutin et al., 2003), leading to an apparent paradox (Misteli, 2001). Note that the self-assembly hypothesis of Misteli, which is generally proposed in the literature, is too poorly described to be tested experimentally, and this paradox still remains to be solved.

Similarly epigenetic hallmarks of euchromatin can be identified. For instance H3 and H4 are hyperacetylated, meaning that the net charge of their tails is reduced, so that the resulting fiber does not fold in a compact state, as was observed by electron microscopy (Garcia-Ramirez et al., 1995). This decondensation hypothesis is often invoked to explain the active nature of this compartment. The list of post-translational modification is not by far exhaustive, but excellent reviews are edited very regularly on this topic (for heterochromatin, see e.g. (Probst and Almouzni, 2011; Richards and Elgin, 2002)).

The crumple globule hypothesis derived from chromosome conformation capture

A number of methods have been established to map chromosome large scale organization based on the capture of spatially adjacent chromatin segments (see section 3c, (Dekker, 2006)). Recent developments, named Hi-C, enable an unbiased identification of chromatin interactions across an entire genome with a precision of ~ 1 Mbp (Lieberman-Aiden et al., 2009). This technique showed that even for distances larger than ~ 200 Mb, the intrachromosomal contact probability is greater than the average contact probability between different chromosomes, suggesting that they are arranged in discrete entities, the so-called chromosome territories (Cremer et al., 2006). Moreover, the intra- and inter-

chromosomal interaction pattern could be decomposed into two compartments, within which contacts were enriched. This dual compartmentalization appeared to be closely associated to the bi-partite organization of chromosomes in euchromatin and heterochromatin. Finally, Hi-C was applied to assess intrachromosomal contact probabilities in human lymphoblastoids, unravelling a power law scaling associated to a slope of -1.08 in the range 500 kbp to 7 Mbp, that corresponds to a spatial range of ~500 nm to 2 μ m. This structural property appeared to be consistent with a fractal organization of DNA in a crumpled globule conformation characterized by a fractal dimension of $f \sim 3$ (Fig. 31). The crumpling was originally imagined to explain relaxation kinetics of polymers rapidly brought in poor solvent conditions (Grosberg et al., 1988). The crumpled conformation is transient, and ultimately collapses into an equilibrium globule, which is the stable configuration in poor solvent. The crumpled globule is not entangled, and large scale loops should be reorganized at a low energetic cost with no need to break physical contacts to liberate genomic sequences. In addition, the crumpled globule favors long range intra-chromosomal interactions, as shown by the power-law dependence of -1 for contact probabilities in comparison to -1.5 for equilibrium globules (Lieberman-Aiden et al., 2009).

A number of models for the large scale architecture of chromosomes have already been proposed and investigated experimentally using *in situ* hybridization of oligonucleotides targeted to specific genomic sequences in fixed cells (FISH). The physical distance between genomic loci (L) was mapped as a function of genomic distances (G) (Munkel et al., 1999; Sachs et al., 1995; Yokota et al., 1995):

$$L \propto G^{\frac{1}{\varepsilon}} \quad (3)$$

with ε the fractal dimension of the line of polymer in space, which is *a priori* unrelated to f (A. Grosberg, personal communication). In the 150 nm–1 μ m spatial range, ε was ~2, and it increased to ~3.2 above 1 μ m (note that a fractal dimension larger than 3 is somewhat surprising, and would deserve further investigations). A confinement for distances larger than 2-3 μ m, which is consonant with the existence of chromosome territories of finite dimension in interphase (Cremer et al., 2006), was recently detected (Mateos-Langerak et al., 2009). Albeit the fact that FISH is an artifact-prone technique that strongly alters chromatin structures smaller than ~1 Mb mainly during the harsh thermal denaturation step (Solovei et al., 2002), different models were built on chromatin loops that were either of ~1 Mbp in length (Sachs et al., 1995; Yokota et al., 1995) or of ~200 Kbp and bundled in groups of ~5 (Munkel et al., 1999). However, their consistency with respect to polymer physics predictions so far remains limited because it was recently demonstrated that confined polymer models could equally well reproduce FISH data (Emanuel et al., 2009).

A new dynamic loop model was recently proposed assuming that the formation of chromosome loops is a random diffusion-driven process, and that loops occur transiently (Bohn and Heermann, 2009, 2010). This model, which leads to the formation of loops of random sizes, relies on two fitting parameters, namely the loop formation probability upon

collision of two chromatin loci and the loop lifetime. Notably, the looping probability is set to low values of $\sim 10^{-4}$ so as to avoid the formation of collapsed, highly entangled, polymer chains. Using an appropriate set of parameters, it was shown that this model reproduces experiments of FISH and Hi-C, as well as the general topography of chromosome territories (Bohn and Heermann, 2010).

Overall the dynamic loop and the crumpled globule models appear to account for experimental data on chromatin large scale organization, though the former is a steady-state model built on transient loops, whereas the latter is kinetically unstable. Despite these differences, it remains unclear whether the structures proposed by Heermann and collaborators (Bohn and Heermann, 2010) or Mirny and collaborators (Lieberman-Aiden et al., 2009) share similarities or not, and future studies comparing their conformations are thus needed.

Diffusion in the nucleoplasm: our proposition of a fractal organization

Structural insights on nuclear organization may be inferred from rheological measurements because nuclear proteins diffuse in the inter-chromatin space, and their motion is hindered by the obstruction of chromatin fibers (Guigas and Weiss, 2008; Saxton, 1993; Weiss et al., 2004). At length scales larger than ~ 500 nm, photoperturbation techniques have consensually demonstrated that diffusion is normal (Beaudouin et al., 2006; Seksek et al., 1997), whereas complex behaviors, which were interpreted in terms of anomalous diffusion or multiple-component diffusion, were observed at smaller length scales of ~ 100 -200 nm using single particle tracking or fluorescence correlation spectroscopy (FCS) (Bancaud et al., 2009; Grunwald et al., 2008; Pack et al., 2006; Wachsmuth et al., 2000).

We observed that the diffusive hindrance and anomalous diffusion exponent γ of GFP multimers containing one, two, five and ten GFPs in tandem were size independent, suggesting that the nucleoplasm architecture is fractal because these structures have no characteristic length scale, so diffusing molecules encounter the same obstructions regardless of their size. Furthermore the fractal dimension of the accessible nucleoplasm could be derived from quantitative modeling of interaction kinetics, revealing that the fractal architecture of nucleoplasm euchromatin and heterochromatin were markedly distinct, and associated to fractal dimensions of $f \sim 2.6$ and 2.2 in the 2-100 nm space domain, respectively ((Bancaud et al., 2009) ,Fig. 31). For a detailed overview on fractal models, see (Bancaud et al., 2012).

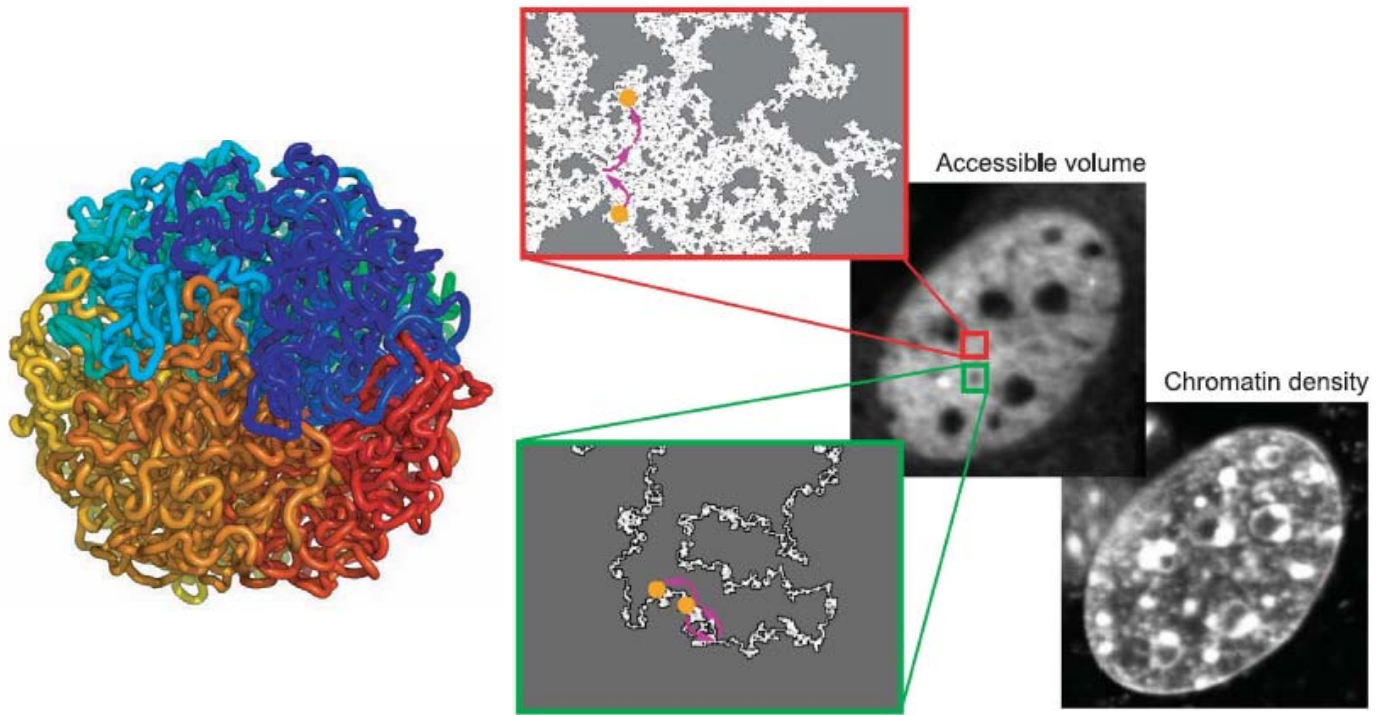


Figure 31 : The left panel shows the simulation of a nucleus containing a polymer folded in a crumple globule conformation, which is obtained by confining a polymer in a compact volume while avoiding the formation of permanent bonds (Lieberman-Aiden et al., 2009). The right panel depicts the architecture of the chromatin-free space in which nuclear proteins diffuse (Bancaud et al., 2009).

Chromosome segmental dynamics: our recent findings on the Rouse model

The details of how chromatin folds and the physical parameters governing its behavior can now be understood by a number of high-throughput technologies (Duan et al., 2010; Lieberman-Aiden et al., 2009). However, the physical parameters governing chromosomes dynamics remain unelucidated, though it has been established a long time ago that the spatial fluctuations of chromosome loci are characterized by rapid dynamics in yeast and in metazoan (Heun et al., 2001; Marshall et al., 1997). More precisely, chromatin dynamics appeared to be determined by nuclear constraints such as the nuclear envelop (Bystricky et al., 2004a; Heun et al., 2001), and by the position of the tracked locus along the chromosome, e.g. telomers (Bystricky et al., 2005). Overall these results pointed to the dynamic character of chromosomes at the molecular level.

The quantitative analysis of these movements has remained controversial for over 15 years, and essentially two models have been used to analyze chromatin trajectories. On the one hand, it has been proposed that chromatin segments undergoes normal Brownian fluctuations at small time scales, and that their motion is confined in volumes of $R \sim 0.3 \mu\text{m}$ (Marshall et al., 1997). On the other hand, the movement of GAL1 locus on Chromosome II appeared to follow an anomalous behavior characterized by an anomalous diffusion coefficient of ~ 0.4 (Cabal et al., 2006a). Interestingly though, these studies were conducted with over a narrow temporal range spanning 1 to 100 s, making it difficult to discriminate

between these two models, which both rely on 3 fitting parameters. Consequently, a mechanistic model describing chromatin dynamics *in vivo* is direly needed.

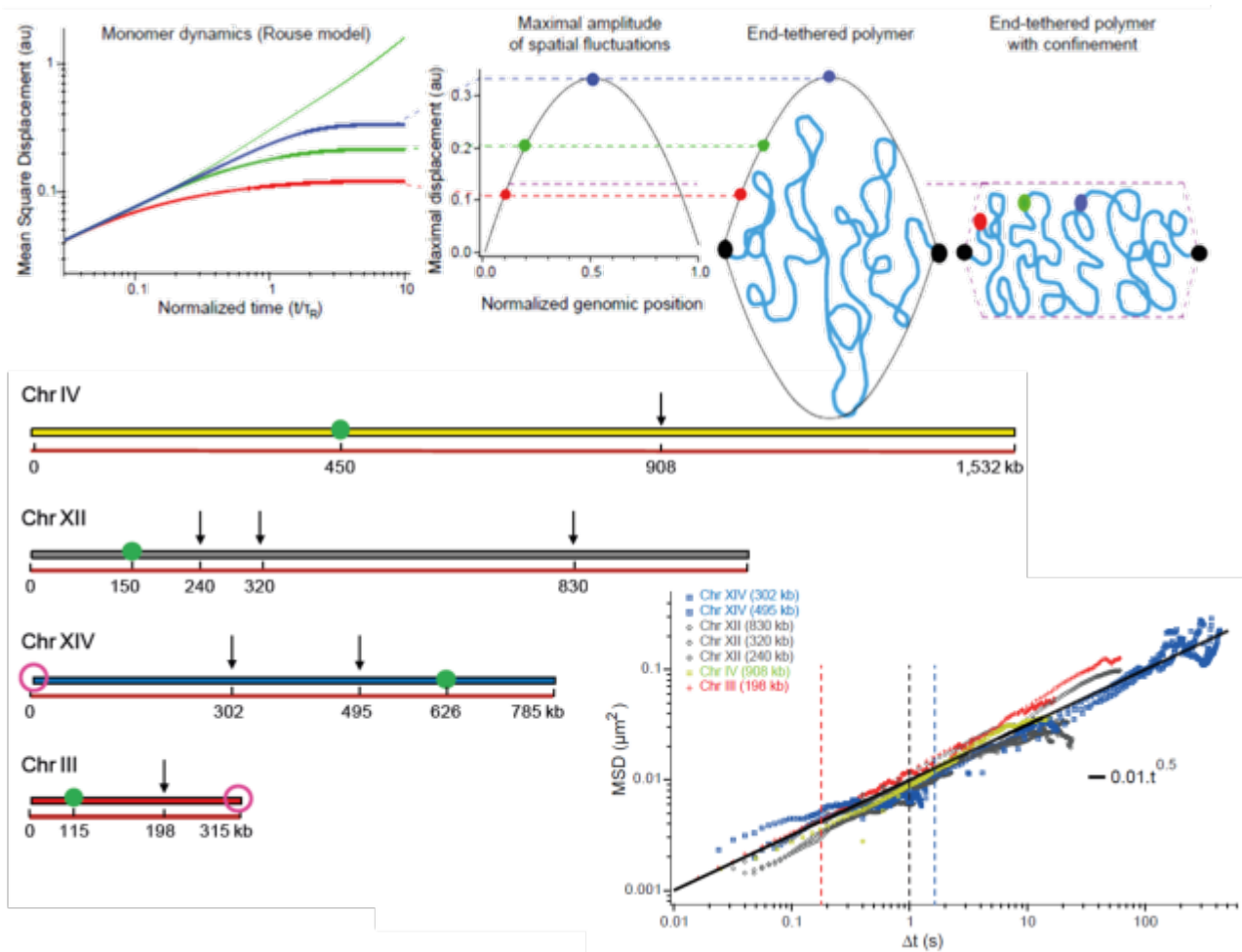


Figure 32 : The upper panel depicts our analytical model to describe the dynamics of chromosomes in living yeast. Two parameters appear to be essential, namely the Rouse timescale and the crowding level that defines the maximal amplitude of the locus displacements. This model was tested by tracking the movements of a series of loci inserted in chromosome III, IV, XII, and XIV, which are characterized by different lengths (**lower left panel**). Our results are shown in the **lower right panel**: the mean square displacement over time for all these loci is consistent with our polymer model of steady fluctuations (publication in preparation).

We recently developed an imaging platform for fast 3D fluorescence microscopy (Hajjoul et al., 2009), and implemented our own image analysis softwares (in preparation). Using this tool we performed high throughput tracking of 9 loci inserted in chromosome III, IV, VI, XII and XIV over an extended temporal range of more than 4 decades (10^{-2} s to 10^3 sec) in *S. Cerevisiae* (Fig. 32). The spatio-temporal dynamics was then confronted to an analytical model that is based on the Rouse formalism with end-tethering and crowding (see text in supplementary material).

We detected a universal response for the Mean Square Displacement (MSD), which was characterized by an anomalous diffusive response consistent with our model. In turn this analysis reveals an unexpectedly high flexibility for yeast chromatin associated to a persistence length of 5-17 nm. Interestingly the Rouse dynamics also implies that

chromosome segments search for target sites by compact exploration (de Gennes, 1982), meaning that they systematically visit neighboring sites as they look for targets during repair or for transcription activation. It would be interesting to evaluate whether and how this compact exploration facilitates genomic transactions using molecular dynamics simulations upgraded with our own particle tracking data.

Our data also indicates that chromatin loci roam a large fraction of the nucleus, and we propose that this broad exploration in the crowded nuclear environment is allowed by chromosome large-scale reorganizations that constantly occur at the timescale of 10-100 s. This dynamic nature is only indirectly detected by Hi-C technologies, which provide a nuclear snapshot averaging chromosome conformations through their interactions. It is in principle difficult to discriminate whether the distribution of chromosome interactions is dynamic or static at the single cell level. Our data in fact suggest that an experiment consisting in extracting the Hi-C map of a single cell at two consecutive time points separated by ~1 minute would lead to very different outputs. In turn we propose that this result accounts for the absence of territoriality in yeast based on the observation that broken ends of chromosomes are free to search the entire genome for appropriate partners (Haber and Leung, 1996).

Finally we note that we are now using our tools for high-throughput motion tracking in living yeast to map the structure and the dynamics of the largest yeast chromosome (XII). For this we mapped the position and the movements of 15 chromosome loci labeled every ~100 kbp along the contour of the chromosome (see text in supplementary material). Localization mapping of chromosome loci with respect to the nuclear envelope and the nucleolus showed the segmentation of the chromosome into 4 domains separated by the centromere and the nucleolus. In addition motion tracking revealed the relatively homogeneous motility of these chromosome loci except for two anchoring positions at the centromere and in the nucleolus, in which a specific spatio-temporal dynamics was detected. Finally, the nucleolar association of some RNA polymerase III transcribed genes indicated that their localization is predominantly determined by the overall chromosome conformation, and marginally driven by transcription factories. Our study therefore puts up a surprisingly simple model of the inner chromosome structure that originates from local anchoring of chromosome arms to specific nuclear compartments, in turn dictating the fate of genes upon biologically driven transactions.

e- Conclusions and research orientations

This section shows that we have studied different facets of chromosomes starting from the DNA to the whole chromosome level. Several conclusions can be drawn from this overview:

- a large body of technologies have been developed to study the mechanics and the biochemistry of DNA, but most of them are not compatible with genome-size manipulation;
- the role of mechanical constraints on the genomic transactions has been extensively studied in vitro, particularly at the DNA level, yielding many speculations that have not been tested in vivo due to the lack of techniques to sense stresses in the genome;
- many questions regarding the structure and the replication of chromatin templates are still open. Yet the tools for the structural inspection of nucleosome arrays are very limited, mostly because these structures tend to interact non-specifically with surfaces, creating adverse effects;
- many tools exist to observe chromosomes in living or in fixed cells, but the models describing their structure and dynamics are too limited to undertake consistent biophysical studies.

Starting from this conclusion I would like to enunciate a few specifications for the development of bio-analytical platforms for sensing and characterizing molecular transactions in the genome. Platforms should perform the following functionalities:

- sense genomic and epigenomic structural information at the chromosome level,
- probe the spatio-temporal dynamics of chromosomes *in vivo*,
- deliver controlled molecular genotoxic stresses, and sensing the response of cells at the population or at the single-cell level,
- comply with the requirements of biodiagnostics to ensure the relevance of our technological developments.

Before digging into our research projects, we wish to clarify a few questions regarding the genome and known diseases in order to convincingly link chromosome disorders to future research in diagnostics.

6. Correlations between genetic / epigenetic deregulations and cancer

a- Mutagenesis and chromosome instability: celebrating a wedding or a divorce?

The characterization of the **anomalous phenotype of cancer cells**, which was first observed by microscopy nearly 150 years at the cytoplasmic and/or nuclear level (Zink et al., 2004), has remained the ‘gold-standard’ for cancer diagnosis for over 100 years (Fig. 33). This phenotypical analysis is qualitative, but image analysis softwares for the systematic registration of phenotypes have been developed, in some cases enabling to collect prognostic information for tumor evolution (Adam et al., 2006).

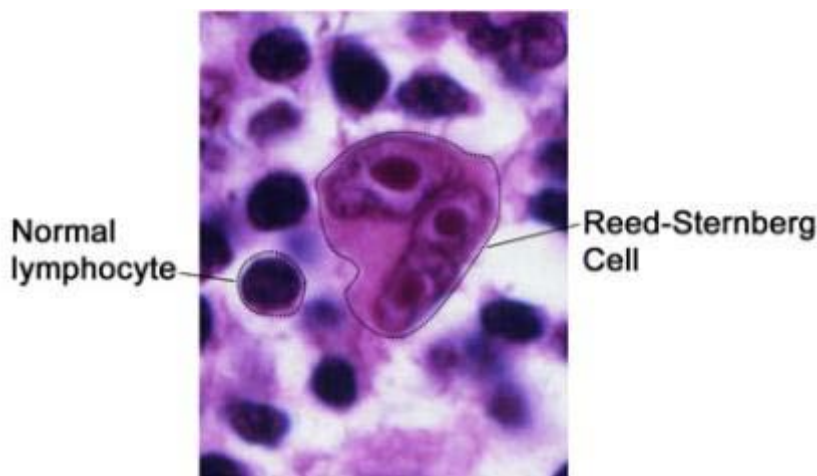


Figure 33 : Use of image analysis for the detection of Adult Hodgkin lymphoma, which is a type of cancer that develops in the lymph system.

In addition to morphological anomaly, cancer cells also exhibited **genomic alterations**, which were first detected under the microscope in 1890 through the form of an excess of chromosomes, a situation coined aneuploidy (Boveri, 1914; Hanseemann, 1890). Genomic alterations were also shown to occur at the gene level through mutations (Morgan et al., 1915; Müller, 1927). In fact the process of tumorigenesis is now seen to emerge from the sequential acquisition of genetic alterations, each representing a bottleneck to the progression into a malignant state. This sequential temporal progression has suggested that tumor development is analogous to **Darwinian evolution**, in which successive genetic changes confer growth advantages to the clonal population (Nowell, 1976). This hypothesis was recently confirmed using single cell sequencing technologies, which revealed distinct clonal subpopulations in tumors that probably represent sequential clonal expansions (Gerlinger et al., 2012; Navin et al., 2011).

The number genetic instabilities still remains unclear: the historical proposition of the “two-hit” model (Knudson, 1971) has been proposed based on the observation that the incidence of retinoblastoma correlates with the random occurrence of genetic alteration events. One mainstream “**mutator**” hypothesis has then been that genetic instabilities should affect genome maintenance genes in the early development of tumors (Loeb, 1991).

Indeed the deregulation of caretaker genes should facilitate further instabilities. Different classes of caretaker genes have been identified, being involved for instance in DNA repair or in cell-cycle checkpoint. Note that the terminology of oncogenes (Weinberg, 1982) and tumor-suppressor genes (Sherr, 2004) is often used, referring to genes involved in proliferation (by e.g. controlling the cell cycle progression), and to genes with repressive effect on the regulation of the cell cycle or promoting apoptosis, respectively.

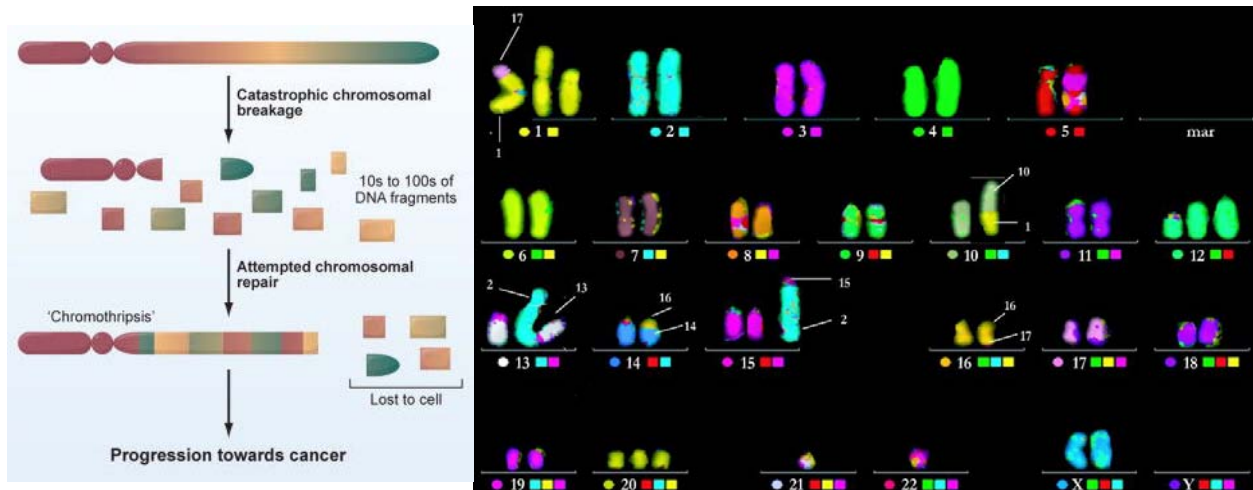


Figure 34 : Schematic representation of the massive rearrangements occurring upon cancer progression in Leukemia (representation is reprinted from (Stephens et al., 2011)). On the karyotype in the right panel, note for instance the anomalous structure of chromosome 13 or 10, which are composed of fragments of chromosomes 2 and 1, respectively.

Importantly recent high-throughput sequencing studies showed an unexpectedly low frequency of mutations for caretaker genes, arguing against the mutator hypothesis for sporadic (non-hereditary) cancer (see (Negrini et al., 2010) for an excellent review). Because large-scale chromosome instability (CIN, for an example see Fig. 34) is present in almost all sporadic cancer, it has thus been proposed that **CIN could be an early event of cancer progression**. Therefore aneuploidy, which has long been viewed as a meaningless consequence of the deregulated growth of tumors, may be essential in tumorigenesis (Duesberg, 2000). Interestingly this hypothesis was tested using a stochastic model, which enabled to compare the kinetics of cancer progression through mutagenesis or CIN as early events (Nowak et al., 2002). This analysis showed that CIN could equally well predict the early development of cancer as mutagenesis. In addition the pursuit of aneuploidy has a strong clinical potential impact because the degree of aneuploidy has been shown to correlate with the severity of the disease (Rajagopalan and Lengauer, 2004; Zhou et al., 2002).

One critical issue of the CIN model is then to identify molecular events responsible for the presence of genomic instability, but it has been argued that oncogenes do not directly induce genomic instability (Cahill et al., 1999). In fact recent models bridge **oncogenes and CIN through "DNA replication stress"** (Halazonetis et al., 2008). Oncogenes

accelerate the cell cycle progression, thus speeding up the rate of replication. However, the ability of differentiated cells to replicate their genetic material is much poorer than for embryonic stem cells, which are programmed to undergo multiple cell cycles in short time-periods (Méchali, 2001). It was shown that genetic instability such double strand breaks can be induced by replication stress in human cells and in yeast (Gorgoulis et al., 2005; Lengronne and Schwob, 2002). Notably this mechanism raises considerable interest because **~80% of common pharmaceutical drugs for cancer treatment interfere with the process of replication**, including among others cis-platinum, 5-Fluorouracile, Gemcitabine, or ara-C which are used for the treatment of lung, colon, pancreas or leukemia, respectively.

Irrespective of how DNA replication stress is induced, double strand breaks do not occur randomly during replication. Rather more than 100 fragile sites, the three most common being FRA3B, FRA16D, and FRA7G, have been identified in the human genome. Fragile sites are identified as chromosomal regions prone to breakage upon replication stress (Sutherland and Richards, 1995). They are known to be late replicating, and the density of replication origins at their vicinity was recently shown to correlate with their fragility (Letessier et al., 2011). In addition they are recognized as hotspots for chromosomal rearrangements in various cancers.

To conclude future researches on cancer development will not necessarily rely on DNA sequencing, and technological developments on whole-chromosome manipulation appear to be well-adapted to tackle questions associated to CIN and their functional consequence on cancer progression. A working model is mandatory to perform quantitative researches, but they are not abundant in the literature. So far we believe that the oncogene-induced DNA damage model is among the most attractive proposition.

In a more general perspective we observe two non-mutually exclusive orientations in the cancer literature. On the one hand a few attempts have been made to bring researches into a “more” logical science, and to identify a small number of underlying principles of cancer progression (Hanahan and Weinberg, 2000, 2011). 6 essential alterations were identified in 2000 (Hanahan and Weinberg, 2000), and this number revised to 10 in 2011 (Hanahan and Weinberg, 2011). On the other hand the complexity of the molecular description of cancer cell deregulations is increasing with the advent of epigenomics, and we briefly overview recent insights in the following paragraph.

b- When epigenomics comes into play

The structural inspection of the epigenetic modifications in chromosomes has also demonstrated the fundamental role of epigenetic alterations in the initiation and progression of human cancer (Jones and Baylin, 2002). The most well documented epigenetic alteration is a global DNA hypomethylation and a promoter-specific hypermethylation at CpG islands (see section 5.a; (Rodriguez-Paredes and Esteller, 2011)). These changes have been commonly described in early-stage tumors, suggesting that

epigenetic deregulations precede the classical genome reorganizations. In fact, epigenetic events can also facilitate genetic damage, as illustrated by the increased mutagenicity of the silencing of the MLH1 mismatch repair gene by DNA methylation in colorectal tumours (Jones and Laird, 1999).

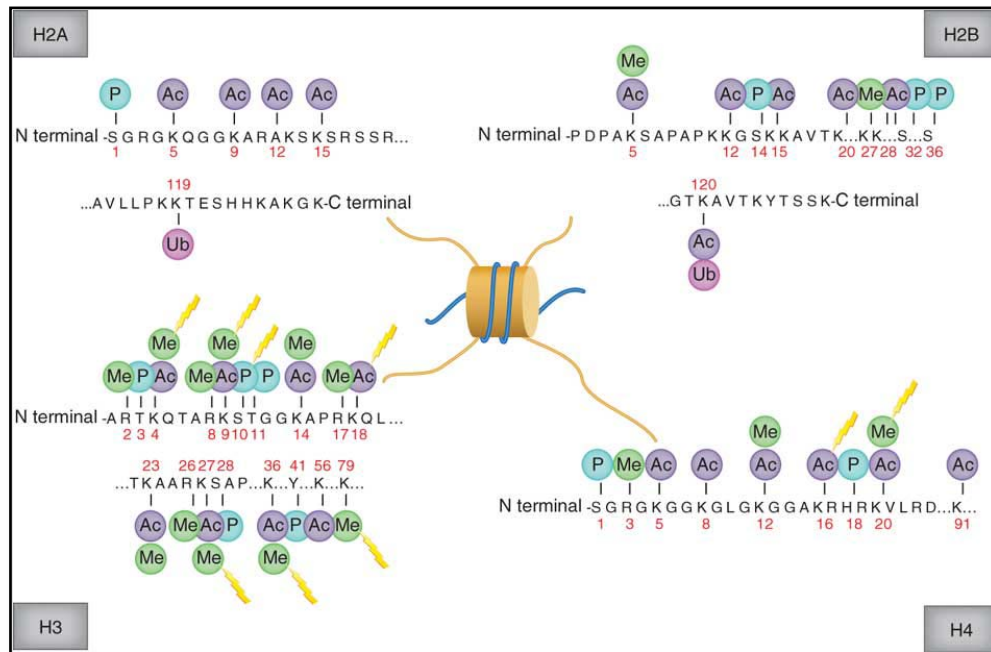


Figure 35 : This figure depicts the main modifications of the four core histones in normal cells (type and position in the amino acid sequence), and it highlights the disruption of normal epigenetic patterns related to cancer. Ac, acetylation; Me, methylation; P, phosphorylation; Ub, ubiquitination. Reprinted from (Rodriguez-Paredes and Esteller, 2011)

Disruption of normal patterns of covalent histone modifications is another hallmark of cancer. To date most covalent epigenetic modifications altered in cancer are located on histone H4 (Fig. 35), including trimethylation of histone H4 at lysine 20 and acetylation at lysine 16 are globally reduced epigenetic imprints (Rodriguez-Paredes and Esteller, 2011). These two specific modifications contribute to establishing condensed chromatin states that turn off gene expression. Overall the subset of genes that are known to be frequently hypermethylated and silenced, or that are often mutated in cancer is limited (Fig. 32), though the documentation of this list is regularly improved owing to the advent of genome-wide technologies.

Because epigenetic mutations are reversible, in contrast to genomic rearrangements, there has been much attention to discover new epigenetic drugs, which could restore the normal epigenetic landscape in cancer cells. So far, four drugs have been approved by the US FDA for cancer treatment: two DNA methyltransferases inhibitors, and two histone deacetylase inhibitors (Rodriguez-Paredes and Esteller, 2011). In addition to cancer treatment itself, the idea of analyzing the DNA methylation map at the genome-wide level in tumor tissues for highly personalized medicine has been growing rapidly. For instance CGIs

hypermethylation analysis has now been used as a tool to detect cancer cells in different types of biological fluids (Shivapurkar and Gazdar, 2010), and considerable efforts are consented to reduce the analytical volumes using next generation sequencing or quantitative PCR (Laird, 2010). In addition, DNA methylation analysis turned out to provide a relevant prognostic marker for the recurrence of lung cancer (Brock et al., 2008).

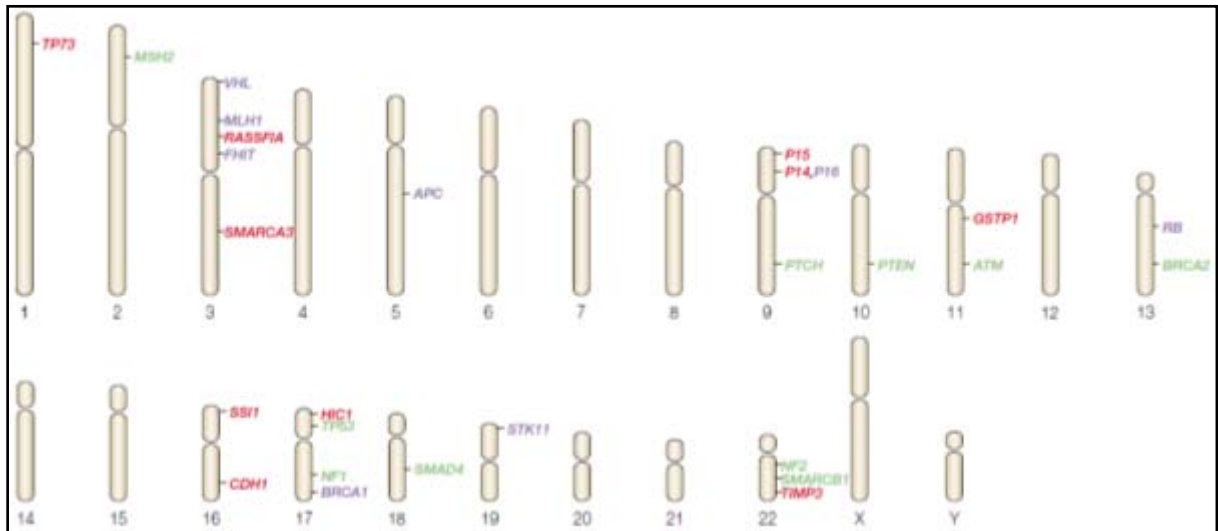


Figure 36 : Representation of the human chromosomes, and the position of the genes that are frequently genetically mutated (green), those that have been reported to be only hypermethylated (red), and those for which both changes have been reported (purple). Reprinted from (Jones and Baylin, 2002).

The complex picture emanating from these studies is that genes can be altered by genomic and epigenomic modifications during cancer. Intriguingly it has been observed that some genes are turned off by genetic alterations, by epigenomic alterations, or by any combination of the two (Fig. 36), and it remains unclear whether epigenetic modifications are anterior or posterior to genomic alterations. This question can hardly be addressed by ChIP technologies, given the cell-to-cell variability of epigenetic modifications and the material required for genome-wide epigenetic profiling. Yet, although sequencing studies have indicated that the mutator hypothesis was unlikely, this epigenetic hypothesis leaves a place for a revisited “two-hit” model, in which epigenetic modifications lead to extinctions of caretaker genes before their physical alterations.

c- Genotoxicity and cancer

Our genomes are constantly challenged by single-strand and double-strand breaks, which occur at a rate of 10^4 lesions per cell and per day due to the presence of $\sim 200 \mu\text{M}$ of oxygen in water at 37°C under air exposure. In addition genomic DNA can be altered after exposure to radiations, such as x-rays, gamma rays, and ultraviolet light, or to chemicals (polycyclic hydrocarbons, acrylamid, metals, anti cancer drugs...). The genotoxic potential of these different substances or radiations has been extensively investigated by a number of technologies that assess the induction of DNA strand breaks, mutagenesis, or aneugenicity

using the COMET assay (Ostling and Johanson, 1984), the Ames test (Ames et al., 1973), or the MicroNucleus assay (Evans, 1977), respectively. Animal testing used to be the ultimate test of genotoxicity in complementarity to these assays based on single cells or unicellular organisms.

Genotoxic analysis plays an essential role in pharmacology, and guidelines are defined to evaluate the genotoxic potential of new drugs. The surveillance of our environment also calls for the development genotoxicity tests. Interestingly despite the amazing detection limits of analytical chemistry technologies, their relevance to field measurements is marginal because they cannot appropriately characterize complex chemical mixtures with strong genotoxic potential.

One objective of genotoxicity is to link long term exposures to disease, in particular cancer. This extrapolation is somewhat difficult, and the correlations between genotoxicity as monitored in single cells and cancer development are not always conclusive. Ironically it should be noted that cancer cells acquire the potential to grow beyond their normal confines, whereas genotoxic tests aim to detect genetic disorders in short time periods that may turn out to be lethal for the cell. Despite this paradox, there is a strong interest in developing modern assays to screen genotoxic agents, and get deeper insights on the link between cancer development and genotoxic stress.

7. Research orientations

a- Motivations

The specificity of my research lies in the variety of my experiences. This background is favorable for cross-disciplinary researches, but it does not define a field of research expertise *a priori*. In this section I describe long term projects that I intend to tackle by novel technological developments (Fig. 37). Four guidelines have oriented my reflexions:

- Micro- and nano-machining offers a unique solution to tailor fluidic systems that perform molecular analysis of limited subsets of cells comprising 1-10 individuals. More generally I believe that **“cell” factory lines can be conceived, enabling to monitor the environment of few cells, study their division under constraints, and ultimately analyse their genomic or epigenomic material at the single molecule level;**

- Most genomic and epigenomic technologies rely on DNA amplification and on bioinformatics, but I think that our technological developments should enable to carry out direct **chromosome structural, hence to minimize amplification and bioinformatic data registration;**

- High-throughput cell factory lines should be combined to high-throughput live cell imaging techniques, because they provide additional molecular information that rely on conventional cell biology assays;

- I intend to set up biophysical experiments, in which **simple observations are analyzed with physics models.**

Three research fields are particularly attracting my attention, namely DNA replication, transcriptional memory, and epigenetic mapping.

- DNA replication is one of the most important steps in the cell cycle, which has been extensively studied to dissect e.g. the molecular events that trigger replication firing. However, there is a huge gap between molecular biology techniques, which infer structural information on e.g. replication factories, and live cell imaging techniques, which provide global information on replication timing. Despite the essential role of replication for cell lineage integrity, there are very few technologies enabling to follow the fate of limited subsets of cells submitted to genotoxic or replication stresses. Therefore, we think that the development of new tools capable of observing the program of replication at the whole-chromosome level and in limited subsets of cells is very relevant.

- Inducible genes in yeast retain a “memory” of recent transcriptional activity, allowing them to be reactivated faster when reinduced after repression (Brickner, 2009). This memory has been correlated with chromatin repositioning within the nuclear space and with chromatin epigenetic modifications at the molecular level. The physical parameters governing this dynamics of chromosomes remain poorly understood, though transcription activation and repression can be monitored with exquisite precision in microfluidic systems, paving the way to an in-depth analysis of this phenomenon.

- There are currently no tools to isolate and scrutiny chromatin fragments of more than ~5 nucleosomes, a ridiculous dimension in comparison to native chromosomes. We therefore believe that new technologies for the analysis of long templates of chromatin are direly needed.

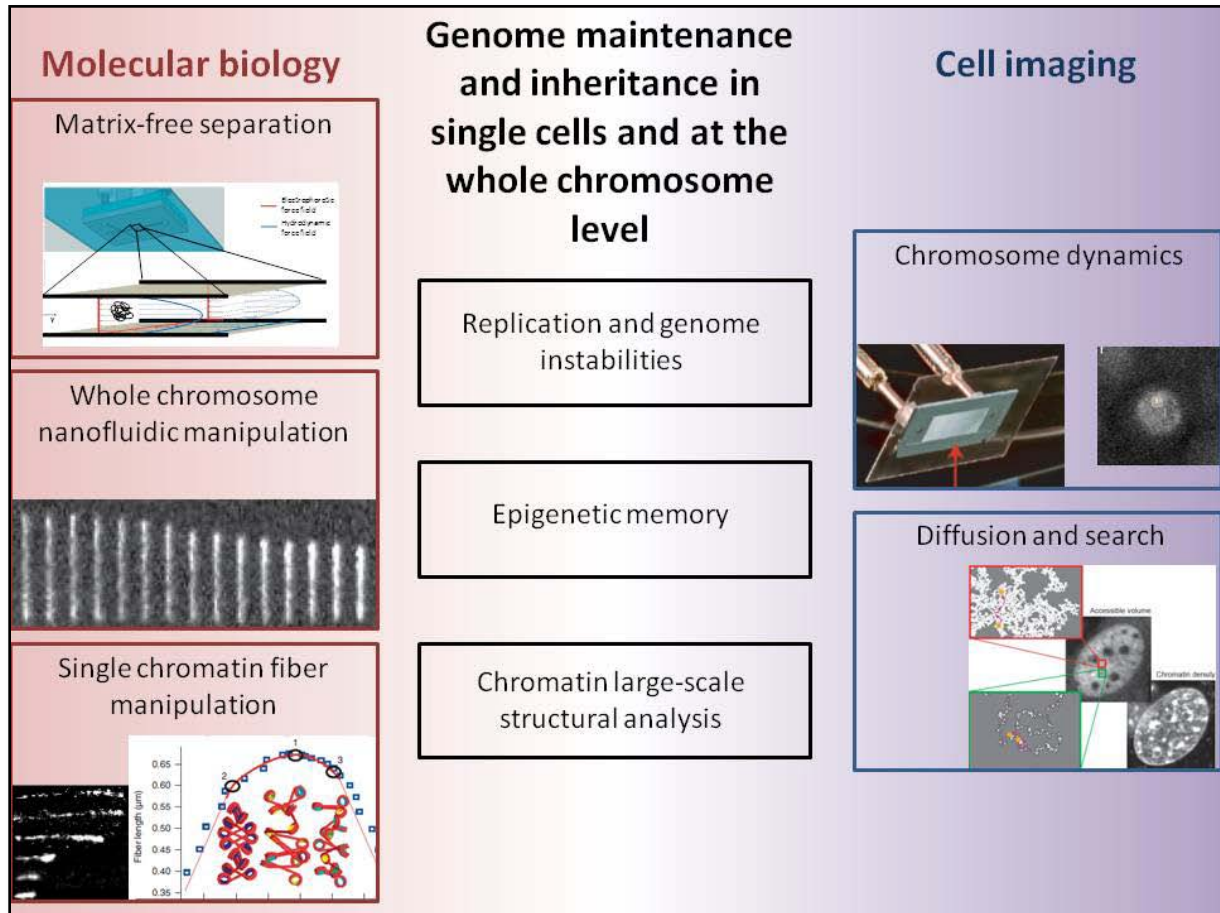


Figure 37 : Overview of some of my previous researches in molecular biology and cell imaging, and the different orientations I envision using this network of expertise.

Finally I mention an on-going research project in “DNA nanotechnologies”, which aims at using short DNA fragments (PCR oligonucleotides) as a cementing material for nanoconstruction. In this case DNA is diverted from its biological function, and seen as an interacting molecule, the properties of which can be controlled with exquisite precision. Notably this “human” use of DNA has become a reality owing to its extensive characterization by molecular biology techniques, as we will explain later.

In this very active research field at the frontiers of biophysical chemistry, inorganic chemistry of nanoparticles, and materials science, we specifically focused on DNA-based energetic materials, and recently demonstrated for the first time the potential of this approach to create highly energetic nanoparticle-based materials (Severac et al., 2012).

b- Chromosome spatial fluctuations and transcriptional memory

State-of-the-art and objectives

The fundamental principles of chromosome organization are still poorly understood, though they raise intriguing intellectual challenges at the frontiers of polymer physics and biology. Transcription of genes is associated to dramatic changes in their physical architecture, which are regulated during development as well as in response to environmental cues. The transcription status of a gene is inherited, and the same gene remembers that it is 'off' in one cell lineage and 'on' in another (Francis and Kingston, 2001).

The transcriptional regulation of the genes involved in the galactose metabolism of budding yeast serves as a paradigm for complex gene regulatory networks. The gene cluster *GAL7-GAL10-GAL1*, hereafter referred to as GAL1, can be found in three major types of regulated states dependent on carbon source: inactive-repressed (glucose); inactive, poised for induction (raffinose); and active, induced to high-level expression (galactose). The GAL1 system raises peculiar interest because the first induction of GAL1 transcription occurs in ~1 h, and this kinetics is considerably accelerated upon reinduction of cells even after long periods of repression spanning up to 5 cell-cycles.

The proteins involved in this regulatory network have been studied, and their interactions in positive or negative feedback loops are also worked out (Acar et al., 2005). The mechanism of transcriptional memory remains discussed, either pointing to the fundamental role of chromatin epigenetics (Brickner et al., 2007), or of signal transduction (Kundu and Peterson, 2010). Interestingly, one key ingredient of the induction pathway appears to be the repositioning of GAL1 from the nuclear lumen to the periphery when raffinose is replaced by galactose in the culture medium, and GAL1 peripheral localisation was shown to be coincident with active transcription (Cabal et al., 2006b; Schmid et al., 2006; Taddei et al., 2006). Moreover, fluorescence microscopy shed light on the complex architecture of chromatin at the active GAL1 locus, which is characterized by two dominant conformations. Thus chromatin structure and dynamics both appear to play critical roles in GAL1 induction pathway, but their exact function has not been understood. **This spatial redistribution of chromatin is a clear biological read-out that can be connected to a polymer-physics model, a situation therefore ideal for biophysical studies.**

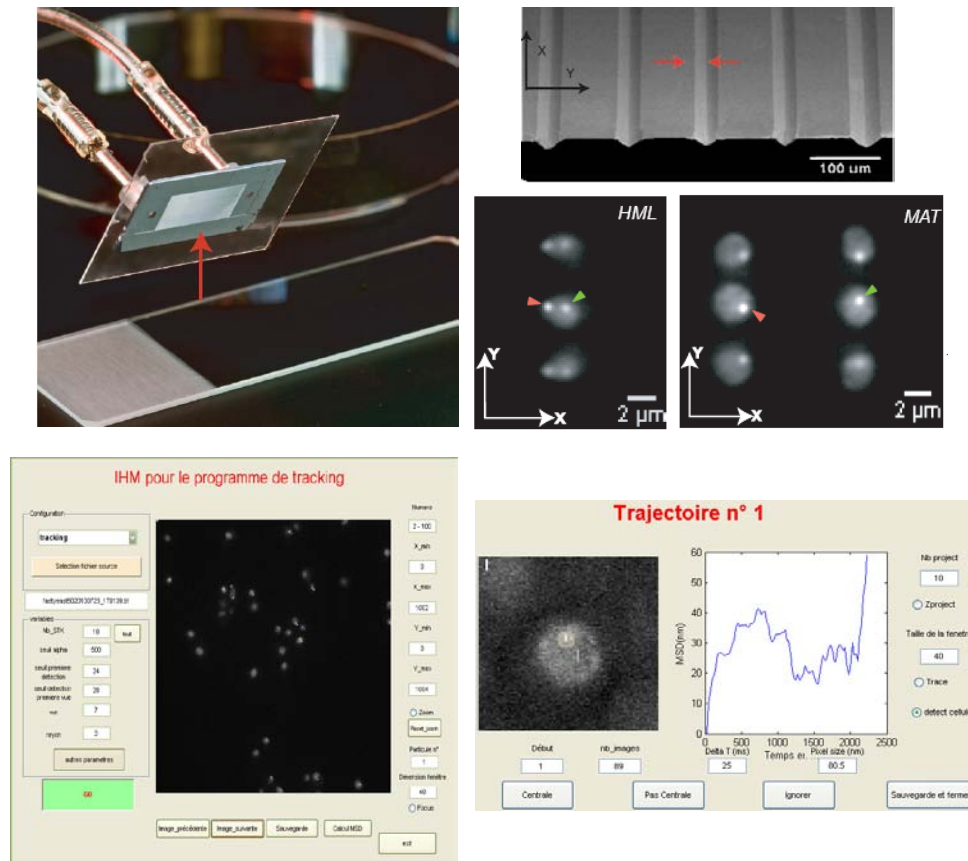


Figure 38 : The upper panel shows our 3D particle tracking technology, which is based on microfabricated mirrors etched in silicon. The picture at the left is an integrated lab-on-chip with two inlets, and the moiré at the center of the chip is a network of V-grooves, which are characterized by electron microscopy in the upper right image. Genetically engineered yeast cells with one or two fluorescently labeled chromosome loci, are placed in the grooves of the mirrors: the central image corresponds to the object, and the upper and lower images are reflected and tilted views of the sample. The lower image is a representation of our software for automatic chromatin loci motion analysis and spatial segmentation.

We recently performed developments for image analysis or 3D imaging to monitor the spatio-temporal dynamics of chromosomes in living yeast (Alber et al., 2012; Hajjoul et al., 2009; Hajjoul et al., 2012; Hajjoul et al., 2011)⁸, and we implemented quantitative models to analyze data collected in living cells (Fig. 38). In addition, we will engineer innovative microfluidic systems for the generation steady galactose/glucose concentration gradients (Dertinger et al., 2001). **Gradient microfluidic systems indeed allow to control the GAL1 pathway in its active or repressed state, or in any combination of the two, and to simultaneously observe chromatin dynamics in real time (Charvin et al., 2010) for a wide range of chemical stimulations (Fig. 39).** These systems will be constructed for the observation of a yeast colony comprising ~300-500 yeast cells trapped in a region of 200

⁸ Note that our work on 3D imaging in living yeast showed us that tracking of multiple chromatin loci was a bottleneck for quantitative data extraction. We thus developed our own software built on the MTT interface (Serge, A., Bertaux, N., Rigneault, H., and Marguet, D. (2008). Dynamic multiple-target tracing to probe spatiotemporal cartography of cell membranes. Nat Methods 5, 687-694.), which was recently used for a training on “advanced yeast imaging” in the 2011 BIAT workshop organized by L. Héliot in connection with the GDR 2588 (MiFoBio).

$\mu\text{m} \times 200 \mu\text{m}$ which corresponds to the field of view of 50 X (NA=1.4) objective. We will use an EMCCD camera with $8 \mu\text{m}$ pixels and 1000×1000 read-out grids (Andor, currently in house), which will allow us to precisely map GAL1 genes relocation upon activation in individual cells. Notably, yeast cells will be trapped at a given position along the gradient and in the focal plane of the objective using a “squeezing” microfluidic system (Gervais and Jensen, 2006). When pressure is released, yeast can be maintained in defined positions in the gradient, and their behaviors can be studied during long acquisitions (Charvin et al., 2010), as proposed by ONIX technologies (Fig. 39). This new technology will improve the reproducibility of our imaging assays. In addition, the possibility to precisely assay cell behaviors upon drug induction for a broad range of concentration may provide insights on the molecular mechanisms that determine chromatin response.

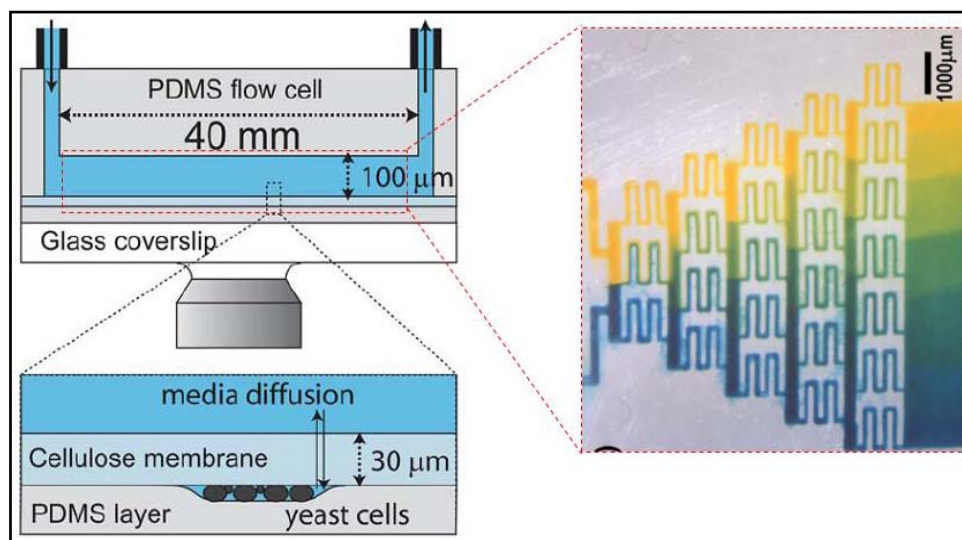


Figure 39 : Principle of the microfluidic device to monitor chromatin large scale reorganization upon expression induction. These tools not only allow for long observations, and subsequent perfusions of galactose and glucose, but also to generate steady gradients, which we intend to use to explore the kinetics.

High throughput chromatin reorganization screening

In collaboration with O. Gadai and K. Bystrycky, we wish to label an inducible locus at its native position. We will initially use the previously studied *Gal1-10* locus (Berger et al., 2008) using galactose induction. We will place its transcription under the control of an ERE (oestradiol receptor element (Bovee et al., 2004; Chu et al., 2009)). Preliminary results from our collaborators, Emmanuelle Fabre (Pasteur Institute), show that $1 \mu\text{M}$ of Estradiol allows rapid induction of the *GAL* gene. Estradiol has several advantages:

- induction is possible without changing the growth media circumventing the side effects affecting nuclear morphology;
- estradiol will only activate the reporter, which offers the possibility to compare results with existing data on endogenous sites;
- reporter cassettes can be inserted near already established labels along the chromosome arm.

We can thus test how far changes of chromatin compaction and dynamics in response to transcription activation reach along the chromatin fiber (2, 10, or 100 kb). What is the duration of the observed changes? Are they immediate or delayed, do they occur in a single hit or in waves? In addition, the labelled *Gal1-10* cassette will be moved to positions that we will select based on our understanding of chromatin compaction to assess the importance of chromosome organisation on transcription activation. Consequently, this project will allow us to **derive quantitative information on the rate-limiting parameters associated to chromatin dynamics in the induction pathway.**

Chromatin reorganization kinetics will then be quantified by measuring the mean relocalization time from the nuclear lumen to the periphery, and when necessary, we will be able to simultaneously assay the transduction pathway response by measuring the level of expression of proteins involved in the network, e.g. GAL80p (Acar et al., 2005) fused to a red-shifted fluorescent protein. The first question we intend to address is whether the GAL1 relocalization mechanism is passive or active. Interestingly the kinetics of first-passage times for diffusion driven problems are now well worked out from a physics point of view (Condamin et al., 2008). Clear temporal signatures depending on the origin to target distance have been described, thus guiding our analysis to unravel the molecular mechanisms associated to gene repositioning. The role of proteins involved in transcription regulation, in chromatin remodelling, which is known to play an essential role in transcriptional memory (Kundu et al., 2007), or chromatin attachment to the nuclear periphery will then be assayed by measuring how reorganization kinetics are affected in yeast mutants. Interestingly, the integration of microfluidics-based temperature actuators (Ross and Locascio, 2002) is straightforward, allowing for generating temperature gradients from 25°C to 37°C. Using temperature-sensitive strains targeted to RNA polymerase II or nuclear pore proteins (*nup49-313*), we will be able to impair essential proteins of the GAL1 response pathway during activation, and thus to provide additional insights on the molecular interactions associated to chromatin relocalization.

Consequently, this project, which relies on technological developments and physical models that we contribute to improve (Alber et al., 2012; Hajjoul et al., 2012), will allow us to **elucidate the mechanisms of chromosome reorganization in time and in space, and throughout a lineage.** Last but not least, this study will be complemented by the nanoscale mapping of GAL1 chromatin with our novel technology based on nanofluidics (see paragraph d).

c- Replication program mapping

State-of-the-art and objectives

Our genomes are constantly challenged by DNA lesions, which, if unrepaired or improperly repaired, lead to genomic rearrangements that are the main causes of cancer. These genomic rearrangements were initially thought to mostly alter genes involved in genome maintenance, but recent findings rather indicate that one driving cause of genome alterations is associated to replication. Replication is one of the most regulated and concerted process in the cell cycle. This essential step occurs just before the cell cycle progresses into mitosis. It requires a rigorous duplication of the genomic material to provide daughter cells with the same genetic information, and insure the integrity of the genome throughout the cell-cycles. The proliferation of cancer cells is associated to an enhanced rate of replication, which is targeted by common pharmaceutical drugs, such as cis-platinum, or ara-C that are used for the treatment of lung, or leukemia, respectively.

Albeit the high incidence of these cancers, the number of assays to evaluate the efficiency of their respective drugs is critically low. Among the technological options that can be envisioned to improve our description of cancer cell deregulations, we posit that new developments for the precise characterization of the replication program are direly needed to produce predictive information for cancer treatment.

So far DNA combing is the only assay for single chromosome manipulation and structural investigation (Fig. 40). DNA combing was pioneered in 1994 (Bensimon et al., 1994), and it consists in adsorbing and aligning single DNA molecules onto microscope coverslips using the spreading forces exerted by a receding meniscus. Elongated molecules can be visualized by fluorescence microscopy, giving access to genomic information at the single chromosome level at a maximal resolution of ~ 700 bp. DNA combing suffers from intrinsic limitations:

1. Its throughput is low, and DNA are difficult to identify due to their random distributions on surfaces,
2. The maximal size of DNA fragments is ~ 500 - 1000 kbp,
3. The combing procedure requires large samples of $\sim 2 \times 10^4$ cells.

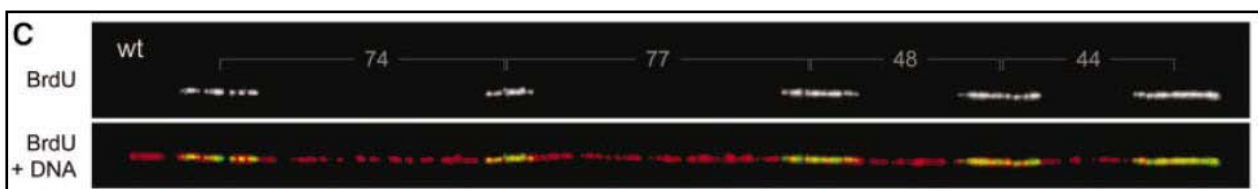


Figure 40 : Chromosomes are purified from cells treated with modified nucleotides, and elongated on coverslips. DNA is stained in red (lower panel), and newly replicated regions in green (upper and lower panel). Numbers indicate distances in kb between replication origins (Reprinted from ref. (Versini et al., 2003)).

DNA combing met considerable success in academic research, in particular to study replication dynamics. Replication is initiated once, and only once, at precise genomic

locations. In metazoans the genome contains $\sim 50 \times 10^3$ origins of DNA replication, that is ~ 1 origin every 10^5 bp. The firing of these origins is finely orchestrated, resulting from the concerted effects of many proteins, and the mechanisms that control this fine regulation remain the subject of intense research. The spatial repartition of replication origins and the timing of replication firing provide a detailed signature of replication, and **this information can be advantageously inferred from optical microscopy.**

Replication origin inter distances, as inferred from DNA combing, are mapped over heterogeneous cell samples that contain at least 10^4 cells. The resulting distributions are broad, and their quantitative analysis by physical models remains mostly disregarded. In fact, the stochastic nature of replication firing (Patel et al., 2005) makes it difficult to link cell-averaged data to single cell dynamics, and this gap cannot be filled with the single molecule techniques available today. In addition population averages make it very difficult to collect relevant data concerning cell duplication and genomic defects inheritance. Chemical, physical or biochemical cues are known to induce dramatic dose-dependent effects on our genomes, but the fate of cells under controlled environment remains hardly accessible. Our objective is to set-up “cell” factory lines adapted to control the molecular environment of 1-10 cells, and to eventually analyze their chromosomes with miniaturized fluidic tools. This research is carried out in collaboration with the group “Regulation of genomic Replication and genetic instability in Cancers” (J.S. Hoffman, CRCT, France), which has a great experience on replication and cancer (Bergoglio et al., 2010; Hoffmann and Cazaux, 2010; Pillaire et al., 2010).

Cell laden for cell proliferation and genome maintenance analysis

My proposal is to use hydrogel laden to manipulate limited subsets of cells, and to use these laden as analytical matrices, interfacing cell biology and molecular biology assays (Fig. 41). Notably this strategy is a miniaturization of our protocols for chromosome purification, which consist in loading 10^4 cells in cm-scale agarose matrices, followed by chromosome purification in this polymer matrix in order to minimize mechanical stress.⁹

Polymer cell laden present several advantages:

- Hydrogels have been broadly used in tissue engineering, and different types of cells can be cultured in cell laden;
- Cell laden can be microengineered using a variety of technologies (Khademhosseini and Langer, 2007), and they can be prepared to accommodate single cells;
- gel matrices are multifunctional materials, encoding photoreticulation, biodegradation, thermal responsiveness properties (Ifkovits and Burdick, 2007);
- chromosome purification can be performed in cell laden;

⁹ The purification of chromosomes takes advantage of the porosity of agarose gels: the size of chromosomes is much greater than the gel mesh size, whereas protein complexes dissolved in SDS diffuse through the gel, and can be efficiently rinsed.

- cell laden can be manipulated using robotic arms, providing a very interesting option to interface macrosystems (96 well plate) with microsystems for molecular analysis.

Convinced by the potential of hydrogel cell laden for 1-10 cell manipulation, we are currently discussing with IMTEK because this institute recently developed a single cell dispenser (Yusof et al., 2011).

The dissolution of hydrogel laden to analyze genomic/epigenomic information stored on chromosomes has to be performed in microfluidic systems. We have developed two complementary technologies, which are compatible with 1-10 cell analysis, namely replication analysis in nanochannels devices and chromosome length measurements in matrix-free separation systems (section 5.a and 4.c, respectively). Note that microfluidics enables to monitor the fluidic environment of biomolecules with exquisite precision, so that physical stress can be minimized in order to avoid DNA breakage. In addition single chromosome analysis is a direct manipulation that does not require signal amplification using e.g. PCR, and it is desirable for future technological developments to minimize the number of steps of analytical protocols.

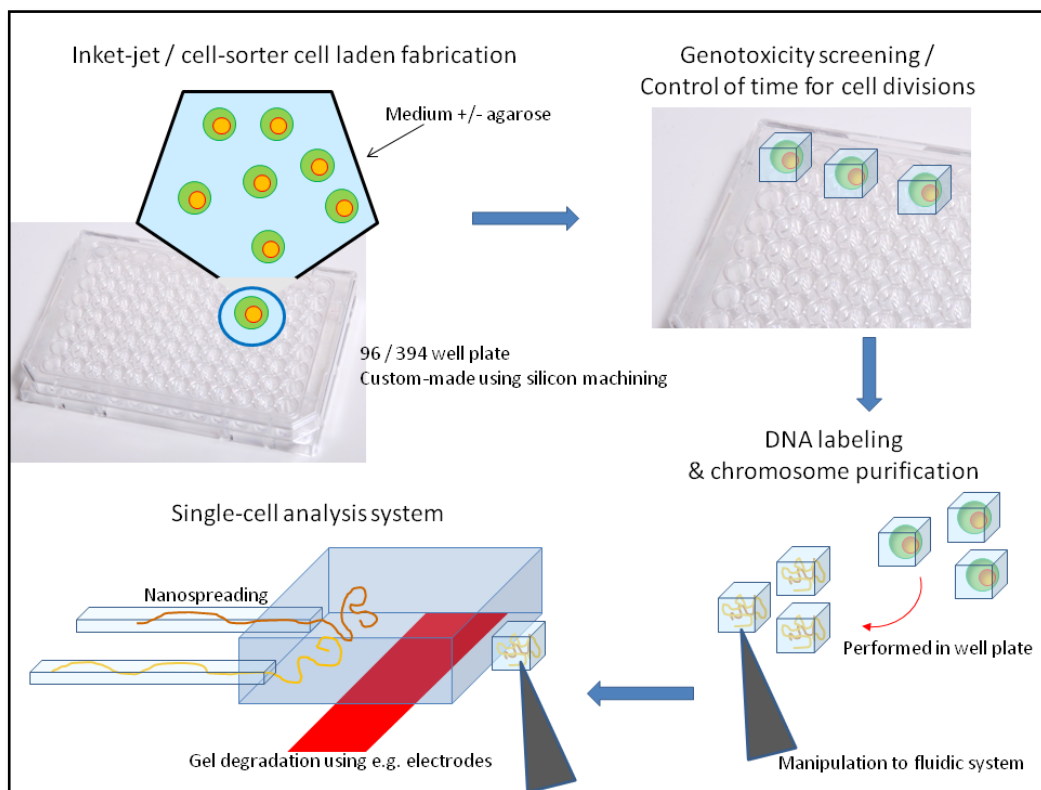


Figure 41: Sketch representing the process to isolate 1-10 cells in hydrogel matrices and analyze their chromosomes. Our proposal involves inkjet/cell-sorter printing of limited subsets of cells, followed by cell-lineage analysis of genome deregulations in 96/394 well plate format. Chromosomes are then isolated, and hydrogel laden serve as platforms for manipulation to micro/nanofluidic systems for chromosome analysis.

This project requires to establish specific protocols to stain newly replicated DNA. We are currently working on this project (RITC funding). We are focusing on the nucleotide analog EdU (Salic and Mitchison, 2008), which is cell-permeable, and enables to label new replicated DNA by click-chemistry (Fig. 42). Notably, despite the consirable success of this method for replication foci labeling in fixed cells, copper is a very strong genotoxic agent, that creates single strand breaks through oxidative damages (Kawanishi et al., 2002).¹⁰ We have recently set up another assay for the direct incorporation of fluorescent nucleotides (Cy3-UTP) by scrape loading (Zink et al., 2003). Although this method likely induces replication stress during the scraping procedure, cells survive this step and can be cultured during several cell cycles, as was demonstrated by our callorators at EMBL (Heidelberg) for live cell imaging of chromatin. Notably the identification of replication origins is generally obtained by dual-color labeling, for it allows to unambiguously assign replication fork polarity. Single-color replication labeling has also been accomplished: the polarity of replication forks was assessed by monitoring the concentration of nucleotide analog over time to generate fluorescence gradients.

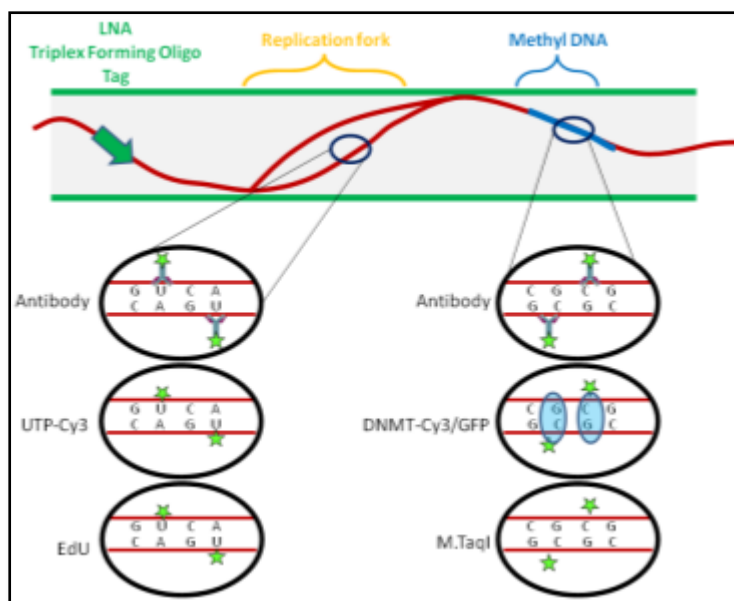


Figure 42 Sketch representing a chromosome (red line) elongated in a nanochannel (green boundaries). We propose to label specific genomic sequences (green), newly replicated DNA (yellow), as well as the distribution of methyl DNA (blue) using various strategies.

Replication-stress, cell division, and fragile sites

We are interested in exploring the process of chromosome instability and its connection to replication stress using the platform described above (Bartkova et al., 2006; Di Micco et al., 2006). The role of oncogenes or of genotoxic agents should be tested, and we find interesting to monitor the fate of cells submitted to continuous stress over several cycles (Fig. 43). This research will involve developments to map **replication progression through fragile sites**. Fragile sites are identified as chromosomal regions prone to breakage

¹⁰ We are currently testing the stabilizing role of glutathione to preserve chromosomes during click chemistry protocols.

upon replication stress (Sutherland and Richards, 1995), and they are recognized as hotspots for chromosomal rearrangements in various cancers. To date, more than 100 fragile sites have been identified in the human genome, and the three most commonly expressed are FRA3B, FRA16D, and FRA7G. These loci are known to be late replicating, and the density of replication origins at their vicinity was recently shown to correlate with their fragility (Letessier et al., 2011).

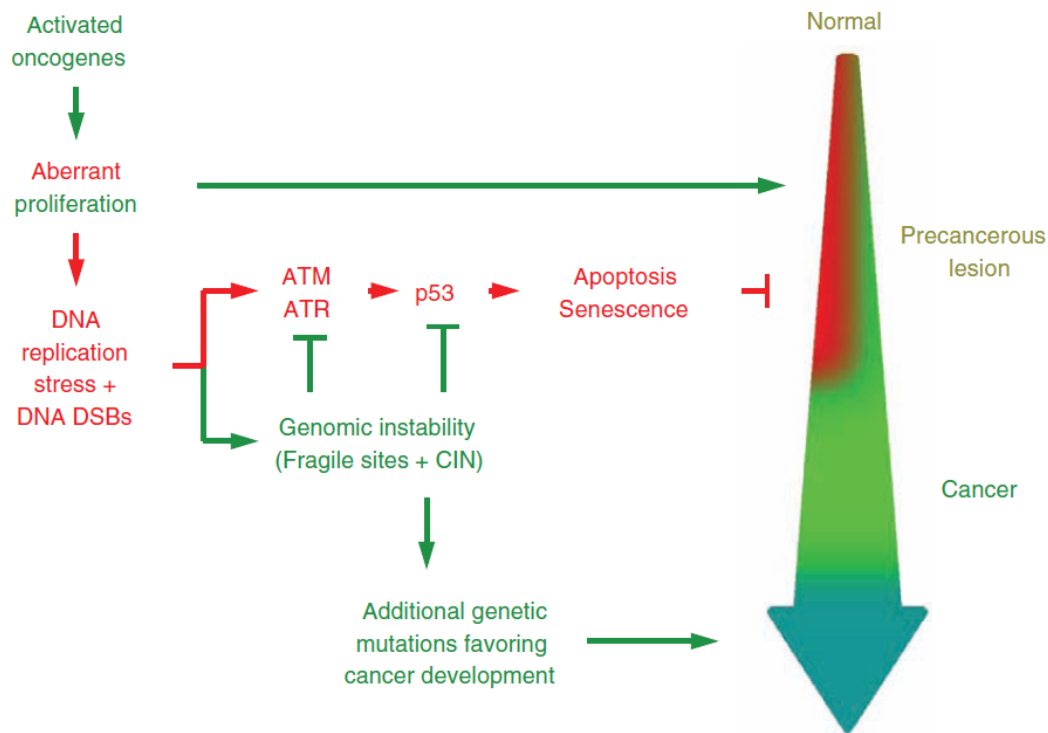


Figure 43 : Model of oncogene-induced DNA damage model involving replication stress (Halazonetis et al., 2008). Note that oncogenes or genotoxic agents may be very interesting to study for this project.

Notably the model of Halazonetis and collaborators (Halazonetis et al., 2008) provides an excellent framework to test how the fate of cells can be altered depending on a minimal number of biological players, and it can be tested using DNA breaks and DNA replication measurements (Fig. 43). In addition given that a high proportion of pharmaceutical treatments interfere with the process of replication, we intend to use our tools for single cell replication analysis with or without fragile site analysis to investigate the effect of common drugs on the distribution of inter-origin distances and on the dynamics of replication fork progression. This project will shed light on **the effect of cancer treatments on replication at the molecular level**, and thus evaluate the replication stress associated to therapies. Moreover, it will enable us to investigate the side effects of pharmaceutical drugs, which are known to be particularly harmful in the context of highly chemoresistant cancer cells.

The labeling of fragile sites requires the targeting of DNA sequences on purified chromosomes (a protocol resembling to DNA *in situ* hybridization). Several possibilities can

be envisioned for this task, and the experience of specialists of this field is necessary. We started discussing with C. Escudé (CNRS, Museum National d'Histoires Naturelles, Paris), who has a long experience in the design of specific probes for genomic DNA mapping for FISH or DNA combing (Escude et al., 2009; Escude et al., 1998), we will optimize tagging methods using triplex-forming oligonucleotides or locked nucleic acids (LNA). To accomplish this project we could first focus on repeated genomic sequences, which provide a strong fluorescence intensity signal. Note that C. Escudé has validated a triplex-forming probe that binds to the centromer of human chromosome 17 (data of FISH and chromosome spreads not shown). Moving to single-copy sequences, which yield low fluorescence signals, may then become very delicate, though the constant improvement of camera sensitivities and fluorophore performances, which are driven by the ever-more challenging specifications of single molecule imaging, will be beneficial to this project.

d- Chromosome epigenetic mapping by whole-chromosome nanospreading

The histone code hypothesis was proposed ten years ago (Strahl and Allis, 2000) based on the observation that histone post-translational modifications and histone variant substitutions constitute inheritable information that plays an essential role in all nuclear functions. **Molecular epigenetics**, which consists in searching for and quantifying epigenetic marks, and in investigating the mechanisms for setting and removing these marks, has been exponentially growing ever since, and it has lead to a dramatically complexified view of chromatin. ChIP and its variants are the main technologies for the physical mapping of epigenetic marks at the genome-wide level. They are however not relevant to precisely assess the heterogeneity of epigenetic imprints in a cell population, because they require abundant samples of at least $\sim 10^3$ cells (Collas, 2010). Moreover the read-length in ChIP analysis is ~ 1 -2 nucleosomes, so that indications on the correlation of the presence of contiguous epigenetic marks along a chromosome are only deduced from a statistical analysis over the cell population. Consequently, beyond the fact that these technologies are labour-extensive, **they leave place for innovative technological developments, and more importantly it remains unclear whether they will constitute robust assays for personalized cancer diagnostics.**

In stark contrast to DNA combing, chromatin combing was never put to work reliably mostly because of the difficulties to monitor the interaction of chromatin fibers with surfaces. We argue that nanofluidics, which is based on maximally repulsive surfaces to allow for the transport of biomolecules in nanochannels, is among the best options to spread single chromatin fibers. These projects have barely started: two studies were carried out on reconstituted chromatin fibers assembled on viral DNA (Streng et al., 2009), or on mono-nucleosomes purified from HeLa cell lines (Cipriany et al., 2010). These two papers are essentially experimental *tours de force*, but they do not yet demonstrate the potential of nanofluidic technologies for chromosome research.

Now, given the number of epigenetic modifications, we intend to focus on two biological questions to validate the relevance of our technology, namely the distribution of epigenetic marks in centromeres and DNA methylation at CG dinucleotides (see section 5.a for details). The level of technical challenge of these two subjects is different, as described in the following sections.

Detection of CpG islands by whole-chromosome nanospreading

Our goal is thus to develop a platform for high-throughput methylation mapping at the whole-chromosome level. Methylation is encoded on DNA, so genomic mapping should rely on developments very similar to those accomplished for replication mapping, except that the labeling of methylated DNA involves different probes.

Fluorescently-labeled DNMTs, and in particular GFP-DNMT, which was recently characterized by P.A. Defossez (Sasai et al., 2010), directly bind to methyl DNA. Note that this labeling approach was used in two recent nanofluidic studies (Cipriany et al., 2010; Fang

et al., 2011), which only mapped methylation on λ -DNA. Engineered methyltransferases were also developed to chemically graft organic fluorophores or chemical moieties to methyl DNA (Pljevaljcic et al., 2007), and this protocol should be directly applicable to purified DNA.

Now, let us evaluate the amount of signal that we expect to collect from one CGI. CGI represent ~ 1 kbp (Takai and Jones, 2002), and they contain ~ 50 CpG. Thus, optimal staining methods should allow to stain one CGI with 50 fluorophores. Given the brightness of modern fluorophores and the sensitivity of microscopy systems, this signal is sufficient for single CGI detection.

The validation of methylation mapping requires to colocalize target genomic sequences and methyl DNA. This could be accomplished using triplex-forming oligonucleotides or LNA. Interestingly, our developments for single analysis in the context of replication can readily be used for methylation mapping, hence allowing us to describe methylation maps at the single cell level, and to assess the heterogeneity of this epigenetic mark in cell populations. Last but not least, the relationship between origin of replication firing and DNA methylation has been recently investigated using genome-wide massively parallel sequencing (Cayrou et al., 2011; Martin et al., 2011), showing that methylation of CpG sequences strongly affected the location of replication initiation events, whereas histone modifications had minimal effects.

Structural mapping of histone-epigenetic marks by nanospreading

Centromeres are the chromosome regions that specify the mitotic behavior of chromosomes because chromosome attachment to the mitotic spindle occurs at centromeres. **Centromeres are defined biochemically by the presence of an H3 histone variant named Cenp-A** (Centromere protein A), which provides the unique epigenetic signature required for microtubule binding. Cenp-A is one of the best-studied epigenetic modifications, but the structure of Cenp-A nucleosomes is still the question of debate (Lavelle et al., 2009), and so is the distribution of Cenp-A and canonical nucleosomes in centromeres. Using conventional microscopy techniques (that is, with a resolution of $\sim 1 \mu\text{m}$), **linearly interdispersed regions of nucleosomes formed of canonical histones or histone variants have been detected** (Blower et al., 2002), but there is a clear need to investigate the molecular structure of centromeres at the nucleosome level to understand how this structure is formed and stabilized. This high-impact project will provide new insights on a key structure for genomic maintenance, as well as consolidate the strength of our technology for biological research.

Purification and spreading of centromeric chromatin

We wish to develop a novel assay for chromatin structure analysis based on nanofluidics. For this, native chromatin fibers will be purified from mammalian cells in order to analyze their epigenetic content by nanospreading. Notably, chromatin is less soluble than DNA, and full-length chromosomes containing intact nucleosomes (and potentially other chromatin binding proteins) cannot be isolated. Nevertheless long chromatin

fragments of 50 kbp, *i.e.* containing ~ 100 nucleosomes, were already purified for e.g. atomic force microscopy studies (Bustamante et al., 1997), and these stretches of chromosomes bear huge amounts of epigenetic information, which is difficult to retrieve with the technologies available today. We intend to collaborate with the group of C. Jaulin (“Epigenetics and Cancer” group, Institute of Genetics and Development of Rennes, France) for centromeric chromatin purification, and with C. Lavelle (Museum National d’Histoires Naturelles, Paris, France) to evaluate the quality of our purification protocols by high-resolution electron microscopy (Dupaigne et al., 2008). Note that we already performed the proof of principle experiment by conveying small chromatin fragments containing ~ 15 nucleosomes inside our own nanochannels (not shown). This preliminary experiment showed that chromatin has a higher tendency to adsorb to surfaces, but still it can be manipulated in nanochannels.

We will envision two methods to fluorescently label epigenetic marks on chromatin fragments (Fig. 44). First, **chromatin can be isolated from cells stably expressing histones fused to fluorescent proteins** (e.g. photoactivatable-GFP), or to chemical tags that allow for the labelling with an organic fluorophore (Wombacher et al., 2010). We note that this approach is particularly relevant for yeast cells, in which genetic engineering allows to fluorescently label every histones. In mammalian cells, the maximal expression level of fluorescent histones reported in the literature is $\sim 30\%$ (Lleres et al., 2009), but the expression of an exogenous pool of fluorescent protein may lead to artefacts associated to over-expression and aberrant phenotypes, which are known to occur with CenpA. In another direction, the growing demand of antibodies for ChIP studies has boosted the demand for highly-specific antibodies, which can be labelled with fluorophores, or indirectly detected with secondary antibodies. **These antibodies can be used to label histones, histone variants, or histone post-translational modifications from wild-type cells** after chromatin purification and before the elongation of the fibers in nanochannels.

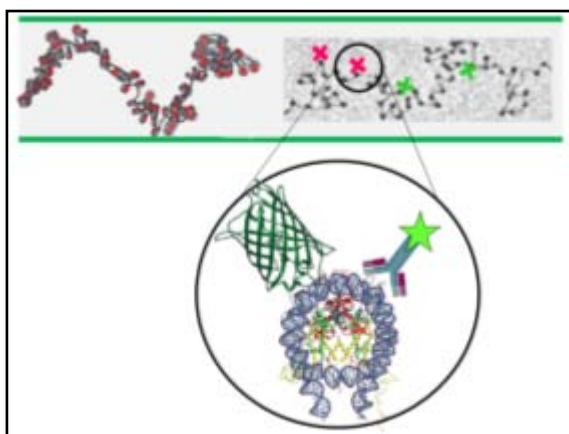


Figure 44 : Principle of chromosome epigenetic structural mapping: 10 nm chromatin fibers are elongated in nanochannels, and the spatial distribution of epigenetic marks, which are fluorescently labeled with antibodies or protein fusions, is detected with nanometer precision using super resolution microscopy techniques.

Optical tools for high-resolution chromosome structural analysis

Single molecules confined in nanochannels have so far been observed by conventional fluorescence microscopy, hence with a spatial resolution of ~ 300 nm, which is larger than

the cross-section of nanostructures. Therefore, despite the exquisite control on the conformation of molecules with nanofluidics, this technology has not reached its full potential for structural mapping. **We wish to use advanced microscopy techniques to bring whole-chromosome nanospreading one leap forward, and reach molecular descriptions of chromosomes.** In our case, we posit that PALM/STORM (Betzig et al., 2006) is a method of choice. PALM consists in stochastically photoconverting fluorophores embedded in a structure from a dark to a fluorescent state, and in measuring the position of these individual probes sequentially in time with nanometer spatial resolution. This technique relies on a conventional bright field microscope, and it can be performed on virtually any chemical fluorophore. It is extensively used in cell biology, but its potential for nanobiotechnologies remains underappreciated: whereas cellular structures spread over $\sim 100 \mu\text{m}^2$, thus requiring long temporal acquisitions to correctly sample the distribution of fluorophores, single molecules confined in nanostructures can be mapped rapidly, as was convincingly demonstrated with the super resolution imaging of DNA nanostructures with two rounds of photoconversion (Steinhauer et al., 2009).

At this point, let us evaluate the spatial resolution required for chromatin structural analysis. Nucleosomes measure $\sim 10 \text{ nm}$, and they are separated by $\sim 10\text{-}20 \text{ nm}$ under low ionic strength (van Holde, 1989). Thus chromatin structural mapping at the nucleosome level requires positional accuracies of $\sim 10 \text{ nm}$, which appear as a challenging yet realistic objective, bearing in mind that sub-nanometer precisions have been reported *in vitro* (Pertsinidis et al., 2010). Note that optimal resolutions are reached in PALM when spatial fluctuations associated to Brownian noise are minimized. Depending on the requirements of our experiments, we will embed chromatin fibers in a gel by photopolymerization. We will most likely use near-infrared initiators (Soppera et al., 2009) in collaboration with O. Soppera (CNRS, IS2M, Mulhouse, France), because we expect to minimize fluorophore bleaching in this spectral domain. Convinced by the unique potential of PALM microscopy and given our expertise on photoactivatable proteins (Bancaud et al., 2009), we recently started to devise a PALM microscope in the host institution through an interaction with M. Dahan (ENS, France). Note that our recent experience in high-throughput particle tracking to investigate chromosome movements in living cells will be useful to carry out this project. Overall, we argue that super-resolution microscopy in combination of nanofluidics will allow us to describe **chromosome architecture with an unprecedented resolution**. Moreover, this research will be the cornerstone to study the distribution of other epigenetic marks at the single molecule level, as well as to **envision new tools for the structural analysis of chromosomes purified from single cells**.

e- DNA-based nanotechnologies

Nature possesses an extraordinary capacity to assemble complex nanostructures that have active and specialized functions. Our ability to precisely position distinct components providing rich functions on the nanometre scale remains a key goal in nanotechnology and materials science. Bioinspired fabrication method has raised considerable interest in the nanotechnology community for it provides a unique way to engineer high-performance and multifunctional materials or systems, thus opening new area in material and electrical engineering.

Among promising bioinspired fabrication method, DNA technologies¹¹ has emerged as one of the most powerful 'bottom-up' approach with a unique possibility to build hierarchical architectures of various nanoobjects, including molecules, nanoparticles, nanoelectronic components..., keeping the fabrication protocol nearly identical. Three decades ago, Nadrian Seeman was proposing to use DNA as a new technological material (Seeman, 1985) due to its remarkable molecular recognition properties. DNA has now come of age, as its folding in 1D/2D/3D is programmable, and it can be further chemically modified for their accommodation on various organic and inorganic substrates. The demonstration of DNA Origami principles (Rothemund, 2006) sets DNA technology as a fertile alternative to pattern nanostructures with an unprecedented level of complexity. Another major property of DNA arises from the ability of small single stranded DNA or RNA –aptamers– selected by directed evolution methods (SELEX) to bind to virtually any given molecular target. Therefore **DNA technologies enable to conduct logical abstraction to design and fabricate an almost infinite variety of DNA based multi-scale hierarchical arrangements of multifunctional materials and nanosystems** with abundant molecules, at relatively low cost. There is no *a priori* fundamental limitation in the design, definition, structure and implementation of industrial processes for the manufacturing of large quantities of DNA based advanced material in the longer term.

There is no doubt that, **bottom-up approach to nanotechnology using DNA will contribute in the next century technological era. They should, in much the same way as silicon in the 1970's, lead to the modern information technology industry with applications expected at addressing challenges in clean energy, environment, biology & medicine and human welfare.**

In this competitive field of research, our contribution is to have demonstrated that DNA-based nanotechnologies could be applied to fabricate high-energy nano-energetic materials. Energetic materials are substances storing chemical energy that can be released by a thermal, electrical, or optical stimulus. These materials are now of deep scientific importance and of wide technological relevance, extensively used for both civilian and military applications. Over the last decade, the particular properties of nanomaterials have

¹¹ Combination of synthetic stable branched DNA and sticky-ended cohesion to structure the matter and object.

led to the fabrication of advanced new energetic compound for explosives or propellant applications (Rossi et al., 2010; Son et al., 2007b). Metastable Intermolecular Composites (MICs) or nanothermite composites (nTC) constitute a relative new class of nanoenergetics with promising performances. They are composed of oxidizer and fuel nanoparticles with typical particle sizes spanning tens to hundreds of nanometers. They are predominantly prepared by physical mixing of powders. Aluminum nanoparticles were mostly studied as fuel, and various oxidizers have been explored including MoO_3 , CuO , Fe_2O_3 and Bi_2O_3 (Bockmon et al., 2005; Foley et al., 2007; Pantoya et al., 2006; Sanders et al., 2007; Schoenitz et al., 2007; Son et al., 2007a). It is usually admitted that the maximum interfacial contact area between the oxidizer and the fuel is desired to achieve optimal energetical performances, although the preparation of nTC by physical mixing does not allow either to control the arrangement of nanoparticles at the nanoscale nor their detailed interfacial properties.

Along this line, the recent development of self assembly techniques to precisely control the nanoparticles or nanoobject assembly in a three-dimensional configuration is of particular importance for nTC. This should result in a precise arrangement of fuels and oxidizers nanoparticles and thus should devise nTC with optimal energetic properties. Zachariah and coworkers (Kim and Zachariah, 2004) proposed an electrostatically enhanced assembly method to synthesize a nanostructured $\text{Al}/\text{Fe}_2\text{O}_3$ nanocomposite. The interaction between nanoAl and nano Fe_2O_3 is favored by oppositely charging each particle in aerosol. They demonstrated an increase in burn rate. Gangopadhyay and coworkers (Shende et al., 2008) have proposed a method to assemble CuO nanorod with nanoAl particles by coating nanorods with poly(4-vinylpyridine) polymer, also showing an increase in burn rate. Finally Yetter and co workers (Malchi et al., 2009) used electrostatic self-assembly based on the functionalization of Al and CuO nanoparticles with oppositely charged ligands in order to create micron-sized Al-CuO spheres.

We recently demonstrated the **synthesis of nanostructured Al/CuO nTC by DNA-directed assembly** (Severac et al., 2012). DNA-directed assembly consists in coating two types of nanoparticles with single stranded DNAs of complementary sequences (Fig. 45), and, upon mixing in aqueous environment, nanoparticles aggregate through DNA hybridization. Since the seminal work of Mirkin and coworkers (Mirkin et al., 1996), the control over the structural DNA length and sequence was shown to provide the tools to generate DNA-programmable NP crystals (Park et al., 2008). Notably, DNA-directed assembly has mostly been applied to assemble gold and silver nanoparticles for bio detection and plasmonic applications (Loweth et al., 1999; Mirkin, 2000; Mirkin et al., 1996; Mucic et al., 1998). While this achievement was obtained with gold nanoparticles, which can be synthesized with precisely defined size distributions and coupled to DNA using thiol moieties, our recent contribution is to have shown the potential of this technology for additional classes of nanomaterials.

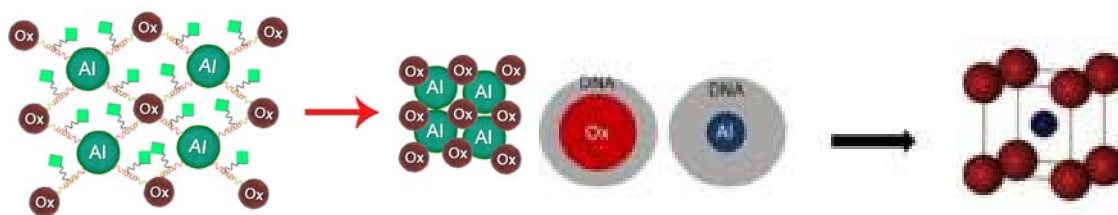


Figure 45 : Principle of 3D assembly of diverse nanoparticles into complex and multifunctional structures.

In collaboration with C. Rossi of LAAS, we are now involved in the demonstration that DNA-assembled energy-generating materials are relevant nanomaterials that can be tuned according to specifications defined during the fabrication process. We aim to validate protocols compatible with the production of real-world products, amounting to improve the reproducibility of bottom-up methodologies, to generate large structures, and to combine these developments with conventional solid-state device mass fabrication.

8. Conclusion

After the revolution in information and telecommunication technology, which dramatically changed our daily environment, much attention has been focused on biotechnologies, which were vaunted as a new frontier generating sustained economic growth and job creation. This scenario is however moving from expectation to skepticism, as for instance outlined by the editorial board of Nature Biotechnology, who wrote a provocative editorial in August 2012 entitled “Can biotech spur job creation?”. In this article they temper the enthusiasm of the Biotechnology Industry Organization 2012 report, abruptly concluding that “urging governments to bet limited public funding on the sector with the promise of job creation might even be downright misleading”.

Despite these critics we have witnessed considerable developments in biotechnologies over the last 10 years, which were obtained using operating principles invented in the 70’s or early 80’s then optimized in throughput using modern electronics, informatics, and robotics. Surprisingly however nano- or micro-technological devices, as obtained in modern clean room facilities, are very rarely used in biotechnologies, albeit their potential for innovative breakthrough is broadly admitted. This gap is also true in biophysics: many researches are conducted with simple fluidic devices (e.g. linear fluidic PDMS network) in combination with elaborate imaging platforms.

Overall I believe that micro- and nano-fluidic technologies should be considered as one essential ingredient of a toolbox also consisting of imaging platforms, robotic systems, computers, and nano-objects. The research projects I proposed in this manuscript rely on this cross-disciplinary association of competences, and they are based on the different knowledge I gained during my research career. They involve an important dose of challenge, but challenges cannot always be overcome. Serendipity and imagination are anyway a major source of innovation, so we should remain optimistic!

To conclude about the skepticism on the future of biotechnologies I would propose the following list of long-term efforts to sustain this activity and improve its economic impact:

- **Education to break the boundaries:** students should be trained to biology and technology to break the walls separating these domains,
- **Physical Modeling:** The term “model” in the biological literature often refers to drawings, which summarize a number of observations. These “models” are not models for a physicist, because they do not rely on quantitative numbers and have poor degrees of prediction. Although this trend is evolving, there is little consensus on how modeling should be carried out, particularly in the field of genomics. Multiscale models, which bridge molecular and large scale interactions in a continuous canvas, are necessary, but they remain to be implemented.
- **Physical chemistry to improve reliability:** molecular biology assays often involve molecular interactions on surfaces with DNA or antibodies as baits. However the

problems of biomolecule-surface interactions are not yet solved, and this limitation considerably limits the transfer of lab protocols to the industry.

- **Design for effectiveness:** new technological solutions implemented by the lab-on-chip community are not designed very rationally, and Computed-Assisted Tools are clearly necessary to speed up the transfer rate. Importantly modeling tools including fluids, biomolecules, and surfaces have yet to be implemented.

These efforts are clearly beyond the scope of my personal career, but some of my researches will hopefully serve as a framework guiding cross-disciplinary researches. As I said in the introduction “my hope is to produce a useful contribution for future researches”, and this sentence constitutes a good ending line for this manuscript.

9. Curriculum Vitae

Aurélien BANCAUD

French, Born December 19th 1976

PhD obtained on November 4th 2004

Married, 2 children

CNRS Research Scientist since 2006 – LAAS-CNRS, Toulouse, France

PhD in chromatin biophysics – with highest honors – 2004, University Paris VI

French Biophysical Society Award in 2005

RESEARCH ORIENTATIONS AS INDEPENDENT PI

Nanofluidics for high-throughput genomic and epigenomic analysis

Advanced imaging technologies for 3D live cell chromatin dynamics visualization

Advanced nanofabrication using DNA as structural material

EDUCATION

2000-2004 PhD in Biophysics, Institut Curie (Paris), November 4th 2004

PhD thesis: Structure and dynamics of chromatin studied by single molecule techniques

2000 M.Sc. in Fluid Mechanics (University Paris VI) with honors

2000 B.Sc. in physics, Ecole Normale Supérieure (Paris)

1997-2001 Ecole Normale Supérieure (Paris) – Major: Physics

PROFESSIONAL EXPERIENCE

2006-present Research Scientist, Laboratory for Analysis and Architecture of Systems, Toulouse - Micro and Nanosystems Department

2005-2006 Postdoctoral Researcher, J. Ellenberg's lab, European Molecular Biology Laboratory, Heidelberg (Germany)

2000-2004 Graduate Research, J.-L. Viovy's group, Institut Curie, Paris

2000 Masters Research, J.-L. Viovy's group, Institut Curie, Paris

1999 Undergraduate research, J. McWilliams' group, Institute of Geophysics and Planetary Physics, University California, Los Angeles

TECHNICAL SKILLS

2006-present Micro-nanofabrication / Micro-nanofluidics / Nanoparticle manipulation / Biosensing

2005-2006 Live cell imaging / Nuclear architecture modelling / Molecular dynamics studied by fluorescence perturbation techniques

2000-2004 Chromatin nanomanipulation / Chromatin purification / Chromatin nanomechanics / Single molecule imaging / Advanced instrumentation

2000 Lab-on-a-Chip / Magnetic particles manipulation

1999 Data processing

MENTORING

2011 J. Lacroix (PhD student 2011-2013)

Nanofluidics for origin of replication mapping

2010 H. Ranchon (PhD student 2010-2012)

Modelling single molecule experiments in nanochannels

2010 F. Séverac (Postdoc 2010-2011)

DNA-based advanced nanofabrication

2009 J. Mathon (PhD student 2009-2012)

Advanced image processing for live cell imaging of chromosomes

2008 Q. He & Y. Viero (PhD students 2008-2011)

Advanced nanofabrication for single DNA molecule manipulation

2007 H. Hajjoul (PhD student 2007-2010, now postdoc in Dijon)

Technologies for high speed 3D imaging of chromosomes

DISTINCTIONS

2005 FEBS fellowship for 2 year postdoctoral fellowship
2005 French Biophysics Society Young Researcher award
2000-2004 Doctoral fellowship of the French Ministry of Research

REVIEWING

ANR Regular expertise for the French research funding agency (2/year)
FOM 1 expertise in 2011 for the Netherlands Foundation for Fundamental Research on Matter
ASF 1 expertise in 2010 for the Austrian Science Fund
OMNT Member of the French strategic watch on Micro and Nanotechnologies (Biotechnologies workgroup) since 2009
C'Nano Regular expert for the French NanoSciences network

FUNDING ID

YEAR	FUNDING SOURCE	PROJECT TITLE	FUND & DURATION
2011	Cancer Research & Therapeutic Innovation Foundation	Replication Lab-on-Chip (coordinator)	1 PhD Oct 11/Sep13
2010	DGA (French Army)	Bio-inspired Nanoenergetic materials (coordinator with C. Rossi)	1 Postdoc + 100 k€ Jun 10/Dec 12
2010	French ministry of Research	Chromatin epigenetic analysis (coordinator)	1 PhD Oct 10/Sep 12
2009	University of Toulouse	High speed 3D particle tracking in living cells (coordinator)	2 PhD + 20 k€ Oct 09/Sep 12
2009	CNRS (Frontier research in physics and biology)	New tools for high speed 3D particle tracking (partner)	60 k€
2008	DGA (French Army)	Nanofabrication for biology (coordinator)	1 PhD Oct 08/Sep 11
2008	ANR (French Agency)	Nanofluidic technologies for genomic analyses (coordinator)	230 k€ Jan 09/Dec 12
2007	CNRS (Frontier research in physics and biology)	Nanofabrication for genomic analysis (coordinator)	60 k€

Citation metrics according to ISI-WoS: 623 citations, H-index = 11

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He (20%), Bancaud (80%) "Method for longitudinal macromolecule spreading and method for analyzing macromolecules" European patent #11306169.1 (September 2011)

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