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Analyzing Chromatin Compaction with FLIM-FRET

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The aim of this thesis project was to design and construct a tool to study chromatin compaction caused by DNA damage response. This was achieved by cloning a bicistronic plasmid, which allows histone H2B fused with two different fluorophores, GFP and mCherry, to be expressed simultaneously in living mammalian cells. Using this plasmid with a fluorescence microscopy technique called FLIM-FRET, the amount of chromatin compaction can be quantitatively analyzed. The project was carried out in a research group led by Maria Vartiainen in the Institute of Biotechnology at the University of Helsinki.

The first step was designing the cloning process and primers needed for PCR. Already existing plasmid backbones were used as basis of cloning. H2B insert was copied by PCR and then cloned into bicistronic IRES vector already containing GFP gene. H2B-mCherry insert was constructed by first cloning H2B into mCherry-N3 vector, and then copying the H2B-mCherry insert into IRES vector. To prevent FRET signal to form within a single nucleosome, H2B was tagged with GFP and mCherry from different ends, leading to a final construct H2B-mCherry-pIRES-EGFP-H2B.

To test the functionality of the construct, a small scale study was designed and executed. Mammalian cells were transfected with the newly constructed plasmid along with suitable controls. Some cells were treated with a chemical trichostatin A (TSA), which is known to cause chromatin to decondensate. FRET signal from these cells was compared to that of non-treated cells.

Based on the test experiment the plasmid appeared to be working to some extent. When comparing FRET signal of TSA treated cells to non-treated, difference was visually noticeable. However, statistical evaluation showed no difference with P-value 0.05. Test experiment had a sample size of 5, and by doing a more comprehensive study, the variance is expected to decrease, leading to more solid results. The plasmid was successful enough to be experimented with in future studies.

Keywords	Chromatin, FLIM, FRET, DNA damage, actin

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Opinnäytetyön tavoitteena oli toteuttaa vektori, jota voidaan hyödyntää kromatiinin pakkautumisen tutkimisessa. Plasmidi sisältää geenit kahdella eri fluoroforilla, GFP ja mCherry, leimattujen H2B-histonien samanaikaiseen ilmentämiseen elävissä nisäkässoluissa. Käyttäen hyväksi FLIM-FRET–mikroskopiatekniikkaa, kromatiinin pakkautumista voidaan kvantitatiivisesti mitata. Työ suoritettiin Helsingin yliopiston Biotekniikan instituutissa Maria Vartiaisen johtamassa tutkimusryhmässä.

Projekti aloitettiin kloonauksen luonnostelulla. Tarvittavat alukkeet käytettäväksi polymeraasiketjureaktioissa suunniteltiin sisältämään kloonauksessa tarvittavat restriktiokohdat. H2B-insertti monistettiin ennestään löytyneestä konstruktista ja liitettiin IRES-vektoriin, joka mahdollistaa kahden geenin ilmentämisen yhdestä vektorista ja joka ennestään sisälsi GFP-geenin. H2B-mCherry tehtiin liittämällä H2B-insertti mCherry-N3–plasmidiin, josta se kopioituiin PCR:n avulla ja liitettiin IRES-vektoriin. Näin saatiin lopullinen konstrukti H2B-mCherry-pIRES-EGFP-H2B.

Vektorin toimivuuden testaamiseksi suunniteltiin ja toteutettiin pienimuotoinen koe, jossa elävät nisäkässolut transfektoitiin juuri valmistetulla konstruktilla ja sopivilla kontrolleilla. Osa soluista käsiteltiin trikostatiini A–kemikaalilla (TSA), jonka tiedetään aiheuttavan kromatiinin pakkautumisen vähentymistä. FRET-signaalia TSA-käsiteltyjen ja käsittelemättömien solujen välillä verrattiin keskenään.

Vektorin todettiin toimivan jokseenkin odotetusti. Visuaalisesti arvioituna käsiteltyjen ja käsittelemättömien solujen välillä havaittiin selkeä ero, tosin tilastotieteellinen analyysi ei osoittanut merkittävää eroa P-arvolla 0.05. Analysoitujen solujen määrä kokeessa oli vain viisi kutakin olosuhdetta kohden ja luultavasti näytekoon kasvattaminen tulevissa koejärjestelyissä vähentää varianssia ja sitä kautta johtaa parempiin tuloksiin. Yhteenvetona vektori todettiin riittävän toimivaksi, jotta sitä voidaan jatkossa ainakin testata erilaisissa koejärjestelyissä.

Avainsanat	Kromatiini, FLIM, FRET, DNA:n korjaus, aktiini

Contents

List of Abbreviations

DDR	DNA damage response
DMEM	Dulbecco's modified Eagle medium, cell culturing medium
DMSO	Dimethyl sulfoxide, used as a cryoprotectant
DSB	Double-strand (DNA) break
EGFP	Enhanced GFP
FBS	Fetal bovine serum, used as a supplement for cell culturing
FLIM	Fluorescence lifetime imaging microscopy, imaging technique where life-time of a fluorophore is measured instead of its intensity
FRET	Fluorescence resonance energy transfer, a phenomena where energy is transferred between two light-sensitive molecules
GFP	Green fluorescent protein
HR	Homologous recombination, a pathway to repair double-strand DNA breaks using unharmed DNA as a template
IRES	Internal ribosome entry site, genomic sequence which allows initiation of translation
NHJE	Non-homologous end joining, a pathway to repair double-strand DNA breaks by ligating break ends directly together
TB	Transformation buffer
TSA	Trichostatin A, an organic compound interfering with histone acetylation
SOB	Super Optimal Broth, a nutrient-rich bacterial growth medium

1 Introduction

This thesis project was carried out at the Nuclear Organization by Actin –laboratory in Institute of Biotechnology at the University of Helsinki. Research group is led by docent Maria Vartiainen and the work was done under the guidance of PhD student Jori Virtanen. The group's research is focused around the role of a protein called actin in the cell nucleus.

One of the cellular processes the actin has previously been linked to is DNA damage response. DNA in nucleus is organized in chromatin, a complex formed by DNA, RNA and proteins. Chromatin can be in compact or in open state (hetero- or euchromatin). The pathways of DNA damage repair are different depending on the state of the chromatin. When chromatin is in its more open shape, the damaged DNA sites are shown to cluster before they are repaired. This process has been previously been showed to involve actin (Aymard, *et al*., 2017).

Studying the mechanisms of this clustering and the role of actin in the process was the main motivation behind this thesis project and the reason why the tool to measure chromatin condensation was needed.

To analyze higher order chromatin structure within living cells a microscopy assay called FLIM-FRET has proved out to be a practical tool (Llères, *et al*., 2009). It has been previously done by generating a stable cell line expressing histone H2B, a protein included in the chromatin complex, fused to two different fluorescent proteins: GFP and mCherry. The aim of this project was to create a single plasmid which could be used to transiently transfect mammalian cells to study chromatin compression by measuring FLIM-FRET signal. This was achieved by using a plasmid containing an internal ribosome entry site (IRES) which allows translation of two separate genes from one plasmid.

2 Background

2.1 Chromatin

In a eukaryotic cell DNA is organized in nucleosomes. A nucleosome is composed of two copies of four different core histone proteins, H2A, H2B, H3 and H4. 145-147 base pairs (bp) of DNA are wrapped around each histone octamer to form a single nucleosome core complex (figure 1). (Luger, *et al*., 1997.)

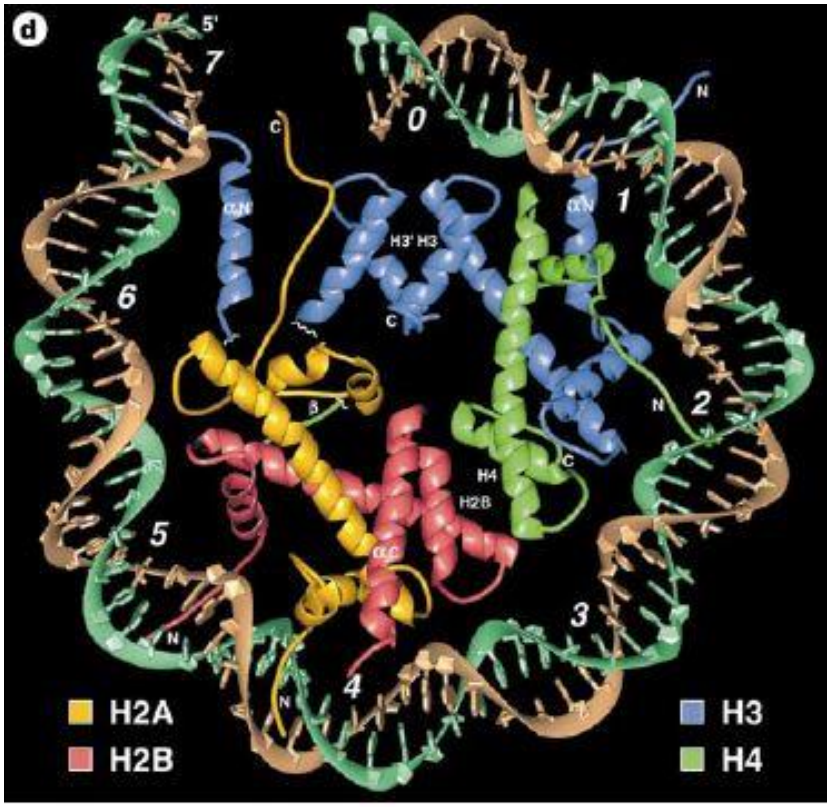

Figure 1. Nucleosome core complex showing DNA ribbon (brown and turquoise) wrapping eight histone protein main chains (Luger, *et al*., 1997).

These complexes shown in the figure 1 are repeated in all eukaryotic genomes approximately in every 200 base pairs. This lightly packed form of chromatin is referred as euchromatin. Nucleosomes can be further assembled into higher-order, more tightly

packed structures called heterochromatin stabilized by the linker histone H1. This packaging of DNA is the main determinant of DNA accessibility within the nucleus. (Luger, *et al*., 1997.)

Chromatin, however, is not a static structure. It has to be mobile in order to make biological events such as transcription possible. Transcription is in general impeded by nucleosomes because the structure blocks access by transcriptional factors into its target DNA. To ensure that the DNA is accessible to necessary proteins, local nucleosome depletion is needed. For example transcriptionally active gene promoters are typically found in a nucleosome free regions. Nucleosomes are not bound to a single position but they can slide along DNA. They are also prompt to disassemble either fully or partially. Furthermore, histones are often modified post-translationally and nucleosome's core histones can be replaced by their variants. Nucleosome positioning and composition are so crucial that the study of nucleosome dynamics can be viewed as the study of genome regulation. (Lai & Pugh, 2017.)

Chromatin condensation can also be modified artificially by using certain chemical compounds, such as Trichostatin A (TSA), a chemical known to interfere chromatin condensation by disrupting histone acetylation, therefore causing chromatin to decondensate (Yoshida, *et al.*, 1995).

Post-translational modifications of histones include phosphorylation, acetylation, methylation and ubiquitination (figure 2).

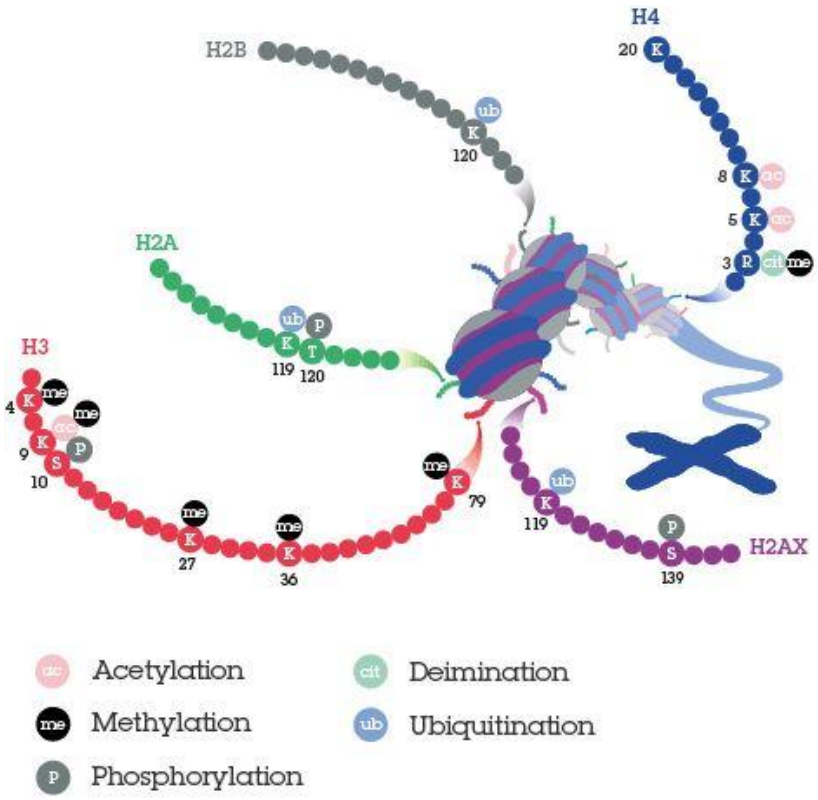

Figure 2. The most common histone modifications (Abcam, 2019).

Most prominent sites for these modifications are tails of H3 and H4 histones. As an example chromatin structure can be directly modulated by altering the charge on histones in a way which reduces interaction between histone tails and negatively charged backbone of DNA. Modifications also affect histones' affinity for other proteins. Histone dynamics is mediated mainly by two families of proteins, histone chaperones and chromatin remodeling complexes. Chaperones are a group of proteins which help histones fold correctly and prevent binding into non-specific targets by neutralizing negative charge of the DNA's sugar-phosphate backbone. Several different histone chaperones exists for different histone variants and pathways. (Talbert & Henikoff, 2017.)

Chromatin remodelers are also a diverse group. These ATP-dependent complexes help for example the proper density and spacing of nucleosomes to be retained, move histones to enable transcription factors to access DNA and makes the histones to be replaced by their variants possible. Remodelers can be classified into four subfamilies and

different classes use different mechanisms of action: imitation switch (ISWI), chromo domain helicase DNA-binding (CHD), switch/sucrose non-fermentable (SWI/SNF) and INO80 (figure 3). (Clapier, *et al*., 2017.)

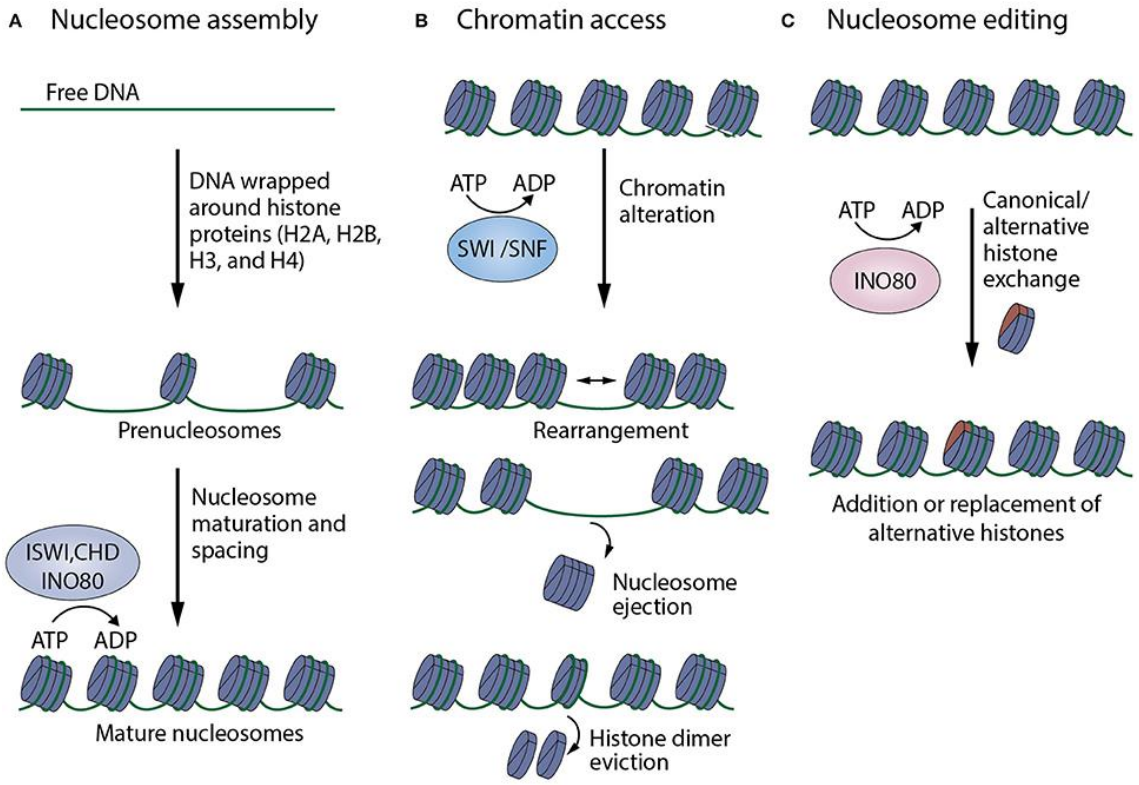

Figure 3. Mechanisms of action of chromatin remodelers (Skopor, *et al.*, 2018).

Figure 3 shows a summary of chromatin remodelers. In nucleosome assembly, factors belonging to the ISWI, CHD and INO80 randomly distribute newly formed nucleosomes. They are also responsible for the maturation and arrangement of these nucleosomes. DNA accessibility is mainly mediated by factors belonging to the SWI/SNF family. Accessibility is regulated by rearrangement, ejection and eviction of histones. INO80 family is responsible for nucleosome editing by promoting the exchange of variant and canonical histones. (Skopor, *et al*., 2018.)

Chromatin remodeling is a very complex process that requires several steps to work properly and it has an essential role in embryonic development by regulating gene ex-

pression. Therefore mutations and other alterations disturbing function of chromatin remodeling complexes can cause several types of cancers and other syndromes. For example mutations in SWI/SNF genes are reported in around 19 % of cancers ranging from stem-cell like cancers to lung cancers. Other examples of disorders linked to remodeler mutations include neuroblastoma and ATRX, CHARGE and Williams syndromes. (Tyagi, *et al*., 2016.)

Not only have histone post-translational modifications and nucleosome organization a major role in transcriptional regulation, recent work has shown that they also influence other processes, such as DNA repair, replication, stemness and changes in cell state. Most studied modifications so far are those that happen on the N-terminal tail regions of histones. As an example when histone H4 has an acetyl group added to its lysine 16 (H4K16ac), the chromatin compaction is reduced and transcription levels are increased. Modifications in the core regions of histones instead of tail regions are an emerging object of studies. (Lawrence, *et al*., 2016.)

2.2 DNA Damage Response

In the scope of this thesis, the DNA damage response (DDR) (reviewed by Hauer & Gasser, 2017) is the most interesting aspect considering chromatin dynamics. DNA is exposed daily to thousands of damage inducing events caused by for example radiation, toxins and free radicals. In order to maintain genomic integrity, DNA has to be effectively repaired. One of the most harmful types of DNA damage are double-strand breaks (DSBs) which are mostly repaired by two opposing main pathways: homologous recombination (HR) or non-homologous end joining (NHEJ) (figure 4).

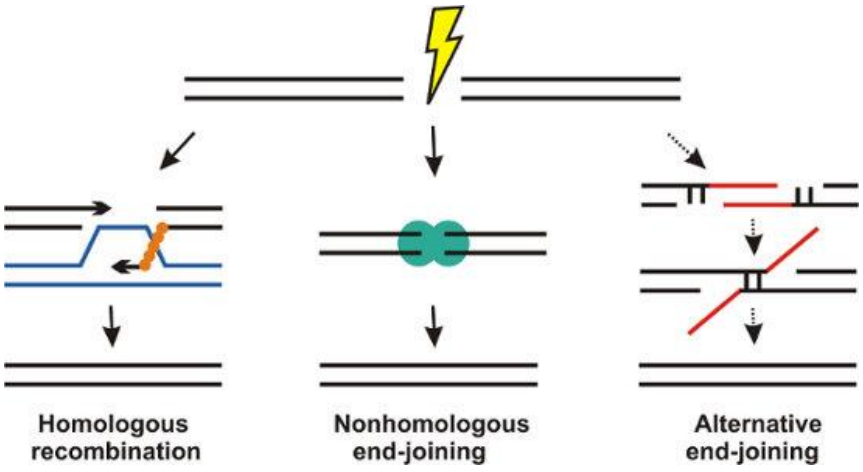

Figure 4. Schematic view of the major DNA double-strand break (DSB) repair mechanisms in mammalian cells (Popp, *et al.*, 2017).

As shown in the figure 4, during homologous recombination genomic information is copied from an intact DNA template and the resulting sequence is usually restored without mutation. In contrast, during nonhomologous end-joining DNA ends are ligated and removal or modification of thermal bases can often cause mutations compared to the original sequence. The repair pathway and efficiency is influenced by initial properties of the chromatin and its position within the nucleus. The formation of DSBs also affects chromatin compaction and DNA motion. It's been proposed that these changes depend on the chosen mechanism of double-strand break repair, for example DNA motion favoring homology search. The importance of the role of chromatin condensation in DNA damage response is backed by studies indicating relaxation of chromatin immediately after DSB followed by secondary recondensation. (Marnef & Legube, 2017.)

Double-strand breaks induced within transcriptionally active genes have been shown to cluster in nuclear space. It was suggested that DSBs in silent genes or intergenic regions are repaired mostly by NHJE, whereas those occurring in active genes are repaired by HR. A study has shown that damaged active genes remain unrepaired and clustered during the first phase of the cell-cycle (G1) and are repaired mostly in postreplicative stage. Mechanism of clustering seems to be mediated partly by MRN complex as well as Formin 2 nuclear actin organizer, suggesting that the process is active. (Aymard, *et al.*, 2017.)

2.3 Actin

Actin is a versatile protein well known to be involved in many cellular processes, such as cell motility and formation of the cytoskeleton. Regulated by actin-binding proteins, it has a unique characteristic to be able to polymerize from monomers (G-actin) into actin filaments (F-actin). Besides being an indispensable protein in cytoplasmic metabolism, actin also has several functions in cell nucleus: regulation of gene expression, chromatin complexes and RNA polymerases. Nuclear actin pool is regulated by different actin binding proteins. Actin is imported into the nucleus by importin-9 and exported by exportin-6. Polymerization is controlled by proteins such as formins and ARP2/3 complex. One of the functions of actin in nucleus is to participate in DNA damage response (DDR). (Virtanen & Vartiainen, 2017.)

Actin can associate with several proteins in the nucleus, some of which have a direct or indirect role in DNA damage response. DNA repair through homologous recombinantion and DSB clustering is shown to involve actin binding proteins ARP2 and ARP3 as well as Wiskott-Aldrich syndrome protein (WASP). These proteins bind at the break site and with actin they promote clustering and homology-directed repair. Schematic figure of different roles of actin in double-strand breaks is shown in figure 5.

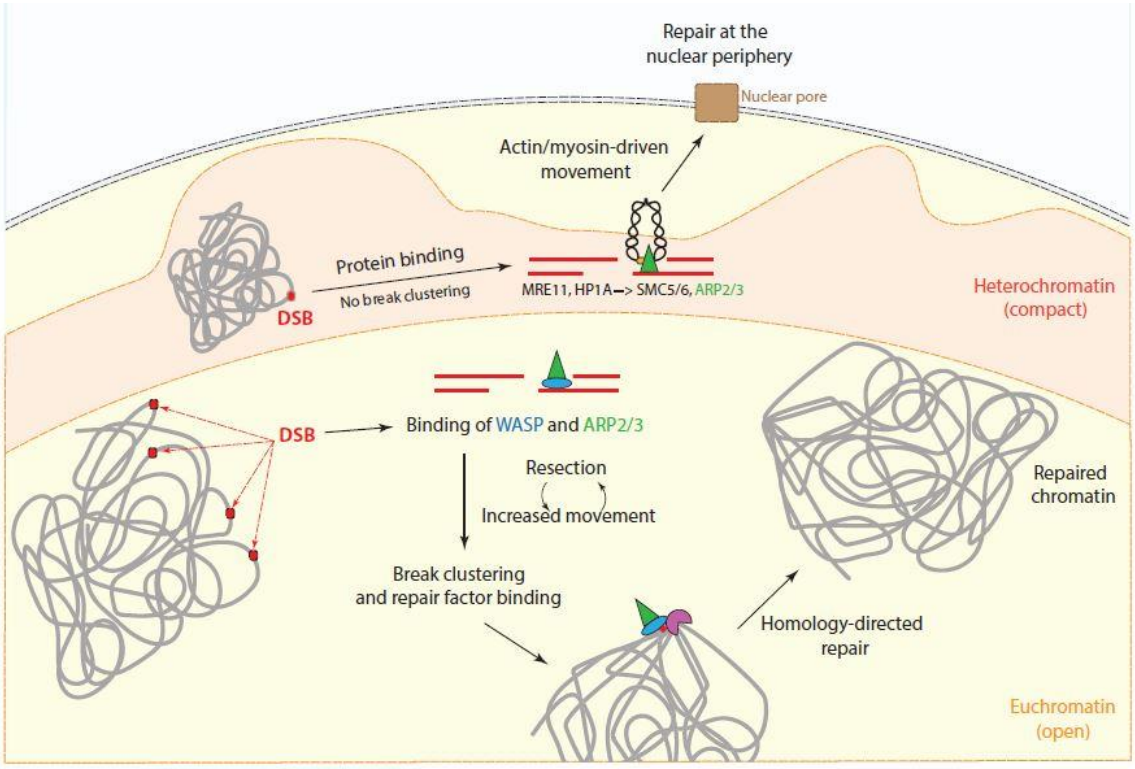

Figure 5. Nuclear actin function in double-strand break (Hurst, *et al.*, 2019).

Figure 5 shows clustering of double-strand breaks in euchromatin and homologous recombination. In heterochromatin break clustering does not happen. (Hurst, *et al*., 2019.)

2.4 Microscopy Techniques

In order to study molecular processes within a cell by microscopy, several fluorescence-based techniques have been developed. Biological samples, such as proteins, can be tagged with different fluorescent proteins. These fluorophores can then be excited by using laser light. When a fluorescent molecule absorbs a quantum of light, a valence electron gets boosted to an orbit of higher energy. After absorbing a photon and entering an excited state, a molecule has several pathways to release its energy and return to the ground state. It can emit a photon, absorbed energy can be converted into heat or the energy can be passed to the molecular environment.

In fluorescence lifetime imaging (FLIM) (reviewed by Becker, 2012), the duration of light emitted by an excited fluorophore is measured. When compared to intensity based measurements, significant advantage in FLIM measurements is that the lifetime depends only on the molecular environment of the fluorophore, not on its concentration.

Fluorescence resonance energy transfer (FRET) is a mechanism where two fluorophore molecules interact with each other so that the energy emitted by the first molecule is transferred to the second one. In other words the emission band of the first one (donor) is overlapping with the absorption band of the second (acceptor). Thus FRET results in a quenching of the donor fluorescence and the donor lifetime is decreased (figure 6).

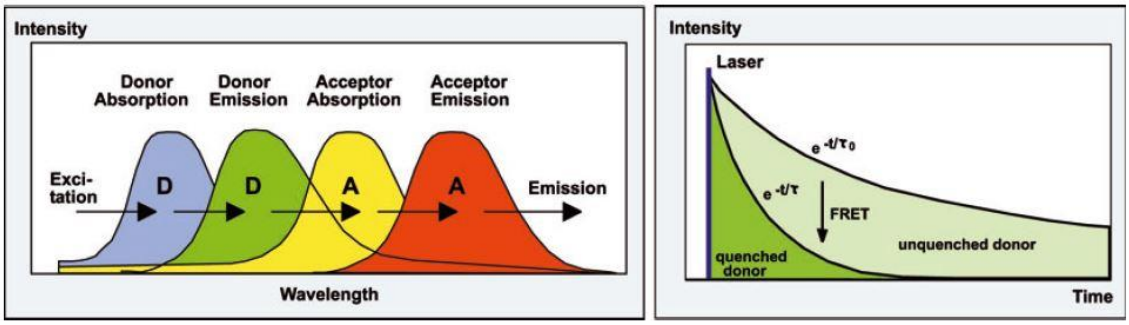

Figure 6. Fluorescence resonance energy transfer. Left: Principle. Right: Donor decay function. (Becker, 2012.)

The energy is transferred by dipole-dipole interaction between molecules and no light emission or absorption is involved in FRET. Rate of the energy transfer is inversely proportional to how far the donor and the acceptor are from each other; it decreases in the sixth power of the distance. For organic molecules FRET is measurable only at distances shorter than 10 nm. (Becker, 2012.)

When FRET is measured intensity-based, the signal is dependent on the concentrations of the donor and the acceptor and therefore the ratio of these two must be obtained. However the acceptor intensity is not directly available due to the overlapping emission bands. This problem can be avoided by combining FRET with FLIM. FRET efficiency can be obtained by measuring fluorescence lifetimes of the donor in presence and in absence of FRET, without contamination from the acceptor. (Becker, 2012.)

3 Methods and Materials

3.1 Construct Design

In order to measure chromatin condensation a plasmid enabling expression of two fluorophore-tagged histones in mammalian cells can be used. After transfecting cells with the plasmid, FRET efficiency can be measured using a suitable microscope. To prevent FRET signal to form within a single nucleosome, histone H2B was tagged with GFP and mCherry from different ends

The frame vector where the pieces would be cloned into was pIRES-EGFP-puro, which was a gift from Michael McVoy (Addgene plasmid # 45567). Plasmid already contains the gene for EGFP. Hence, to get the EGFP-H2B part downstream of the IRES sequence the puro site (puromycin resistance gene) would be digested out and replaced with H2B. H2B could be amplified from an already existing plasmid by PCR.

In order to get the H2B-mCherry construct cloned into the multiple cloning site in front of the IRES sequence, a plasmid with that combination had to be cloned. That would be achieved by amplifying H2B and cloning it into an already existing mCherry-N3 plasmid (mCherry-N3 as well as H2B-paGFP are based on pAcGFP1-N3 frame (Clontech, cat. no. 632484)). By amplifying resulting H2B-mCherry and adding it into the IRES plasmid, the end result H2B-mCherry-pIRES-EGFP-H2B would be achieved. The outline of the design is shown in the figure 7. Detailed diagrams of the frame plasmids with restriction sites are shown in the appendix 1.

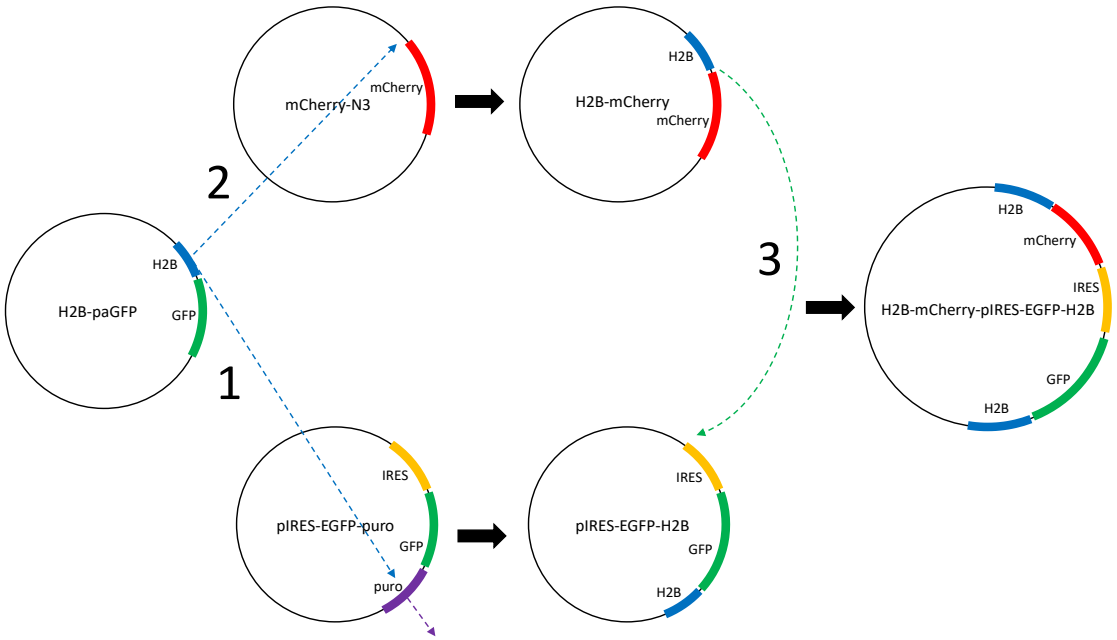

Figure 7. Outline of the cloning procedure.

The procession of the cloning can be divided into three steps as shown in the figure 7:

1. H2B into pIRES-EGFP vector
2. H2B into mCherry vector
3. H2B-mCherry into pIRES-EGFP-mCherry vector

To prepare necessary inserts, previously made plasmids can be used as templates. Inserts with proper cloning sites at the ends need to be prepared using PCR with specially designed primers. Primers are designed to include the following parts:

- **leader sequence** (TAAGCA), this sequence of extra base pairs at the 5' end of the primer assists with the restriction enzyme digestion since many restriction enzymes have trouble cutting DNA efficiently at the end of a linear piece,
- **a restriction site** carefully checked to ensure that there aren't any identical sites found inside the insert or elsewhere in the template,
- **hybridization sequence,** 18-21 base pairs long region, which binds to the sequence to be amplified.

For the reverse primer the reverse complement has to be used to get PCR amplification.

3.2 Imaging Design

To test the feasibility of the newly cloned plasmid, a small scale proof-of-concept experiment was designed. The plan was to transfect mammalian cells (NIH/3T3, ATCC® CRL-1658™) with the plasmid and then treat them with Trichostatin A (TSA). When comparing FLIM signal from TSA treated cells to non-treated, a difference should be noticeable. FLIM signal from cells transfected with H2B-GFP, GFP-mCherry and H2B-GFP + soluble mCherry would be measured as controls. GFP-mCherry is used as a positive control as it should have the strongest FRET efficiency while H2B-GFP should have practically none. H2B-GFP + soluble mCherry was expected to show slight FRET effect.

3.3 Cloning

Cloning was done in three steps after the initial H2B insert was made. All the primers designed and used in PCR are shown in the table 1.

Table 1. PCR primers. Restriction sites are highlighted with red color.

Number	Primer	Sequence
V1859	*Kpn2I*-H2B-*XbaI* fwd	TAAGCATCCGGAATGCCTGAACCGGCAAAATC
V1860	*Kpn2I*-H2B-*XbaI* rev	TGCTTATCTAGATCACTTGGAGCTGGTGTCT
V1711	*XhoI*-H2B-*HindIII* fwd	TAAGCACTCGAGATGCCTGAACCGGCAAAATCC
V1712	*XhoI*-H2B-*HindIII* rev	TGCTTAAAGCTTGCTTGGAGCTGGTGTACTTGGTGAC
V1861	*NheI*-H2B-mCherry-*SacI* fwd	TAAGCAGCTAGCGCTACCGGACTCAGA
V1862	*NheI*-H2B-mCherry-*SacI* rev	TGCTTAGAGCTCGCGGCCGCTTACTTGTACA

3.3.1 H2B Insert

Kpn2I-H2B-*XbaI* insert was amplified by PCR from previously prepared template plasmid H2B-eGFP-N3. Primers were designed to include restriction sites as shown in the table 1 and ordered from Sigma-Aldrich. PCR reaction was performed using reagents by Thermo Fisher Scientific. Volumes used for the PCR reaction are shown in the table 2.

Table 2. PCR reaction volumes.

Volume, µl	Reagent	Catalog number
10	10 X Phusion buffer GC	F519S
1	dNTP Mix (10 mM each)	R0191
2.5	Forward primer (10 µM)	
2.5	Reverse primer (10 µM)	
1.6	Template DNA (500 ng)	
0.5	Phusion Hot Start II DNA Polymerase (2 U/µL)	F549S
31.9	Nuclease-free water	R0582
50	**Total volume**	

Reaction was performed using MJ Research PT-100 thermal cycler with the parameters shown in the table 3.

Table 3. PCR program.

Temperature, C	Time, min	
98	3	
95	0.5	X 28
62	0.5	
72	2	
72	10	
4	∞	

PCR product was purified using 1.2 % agarose gel.

The PCR product was stained by adding 10 µl on 6x gel loading buffer (Jena Bioscience, cat. no 254) into the 50 µl sample and pipetted to the gel next to 12.5 µl of 100 bp ladder (NEB, cat. no N3231S). 1x TBE was used as a running buffer in the agarose gel run, which was carried out with 80 V for 30 minutes. Band from the gel was cut out and the DNA was extracted from the gel using GeneJET Gel Extraction Kit (Thermo Fischer Scientific, cat. no K0692). Final elution was made with 35 µl of sterile water.

3.3.2 H2B into IRES Vector

The purified PCR product (*Kpn2I*-H2B-*XbaI*) and the recipient vector (pIRES-EGFP-puro) were digested using FastDigest restriction enzymes *XbaI* and *Kpn2I* and FastDigest Buffer (10X) (Thermo Scientific, cat. numbers ER0681, FD0534 and B64 respectively). Reaction volumes used are shown in the table 4.

Table 4. Reagent volumes for digestion.

Insert	**Vector**
17 µl PCR product	3 µl plasmid (4500 ng/µl)
2 µl FastDigest Buffer (10X)	14 µl sterile water
0.5 µl XbaI	2 µl FastDigest Buffer (10X)
0.5 µl Kpn2I	0.5 µl XbaI
	0.5 µl Kpn2I
20 µl total	**20 µl total**

Reaction mixes were incubated for one hour at 37 °C. Digested DNA fragments were isolated by gel purification using the method described earlier.

For the ligation 17 µl of purified digested insert and 5 µl of digested vector were mixed together with 0.5 µl (200 units) of T4 DNA Ligase and 2.5 µl of T4 Ligase Reaction Buffer (10X) (NEB, cat. numbers M0202S and B0202S). Reaction was incubated for one hour at room temperature.

Ligation mixture was transformed into One Shot™ TOP10 Chemically Competent *E. coli* cells (Invitrogen, cat. no C404006). Cells were first thawed on ice (15 min). After that 12.5 µl of the ligation mixture was added with cells into a round bottomed 15 ml tube and incubated on ice for 30 min. Cell mixture was then treated with heat shock (30 s in 42 °C water bath) and kept on ice for two more minutes. 900 µl of LB media was added into the tube after which it was incubated for one hour at 37 °C in a vertical shaker. To reduce liquid volume before plating, the cell mixture was centrifuged for 5 min at 1600 rpm and the supernatant was discarded. The pellet was resuspended in a small amount of liquid left in the tube and spread on an LB agar dish with 100 µg/ml ampicillin (ICN, cat. no 190148) for selection.

Transformations were grown over night at 37 °C. Four colonies were picked randomly from the plate for DNA isolation. Thermo Scientifics GeneJet Plasmid Miniprep Kit (cat. no 5810R) was used for isolating DNA. Purified plasmids were eluted with 50 µl of sterile water.

To check if the cloning had worked, test digestion was performed for the four minipreps with the same *Kpn2I* and *XbaI* enzymes used for the cloning. Test digestions were done in total volumes of 20 µl: 5 µl of sample, 2 µl of buffer, 0.5 µl each restriction enzyme and 12 µl of sterile water. After one hour incubation at 37 °C samples were run on an agarose gel. Test digestion showed no cutting of any of the replicates, which indicated that the cloning had not worked properly. After some research the problem turned out to be Dam methylation of the *XbaI* restriction site at the destination vector. To circumvent this problem *dam*$^{-}$ *E. coli* K12 genotype was ordered (NEB, cat. no ER2925). After making these cells chemically competent (see chapter 3.4), the cloning procedure was repeated with new bacteria strain successfully.

3.3.3 H2B into mCherry Vector

To clone the H2B-mCherry insert into the IRES vector, the construct with H2B-mCherry had to first be cloned. It was done by amplifying *XhoI*-H2B-*HindIII* insert with PCR and adding it into a destination vector mCherry-N3. Template vector for PCR reaction was H2B-paGFP, a previously made plasmid. Reaction was performed identically to table 2, using proper primers. PCR program is shown in the table 5.

Table 5. PCR program for XhoI-H2B-HindIII.

Temperature, °C	Time, min	
98	3	
95	0.5	X 28
65	0.5	
72	0.5	
72	10	
4	∞	

DNA purification, digestion (with NEB *XhoI* and *HindIII*, cat. numbers FD0694 and FD0504) and ligation was done in the same manner as previously described. Transformation was made into TOP10 cells. Four miniprep replicates were prepared and a test digestion was done with *XhoI* and *HindIII*.

3.3.4 H2B-mCherry into IRES-EGFP-H2B Vector

Final step of the cloning process was adding the H2B-mCherry into the IRES-EGFP-H2B. This was done by amplifying the insert *NheI*-H2B-mCherry-*SacI* from the plasmid made in the previous step by PCR using designed primers. Protocol was as previously, using 65 °C as annealing temperature and 1 minute elongation time. Insert and the recipient vector were digested with *NheI* and *SacI* (Thermo Scientific, cat. numbers FD0973 and FD1133) and ligated as before. Ligation was transformed into TOP10 cells and minipreps from six different colonies were prepared. Final test digestions were conducted with restriction enzyme pairs *NheI-SacI* and *NheI-Kpn2I* to cut out H2B-mCherry and H2B-mCherry-IRES-EGFP respectively.

3.4 Making Competent K12 Cells

To make chemically competent cells to be used for transformations a colony of K12 cells was grown over night in 10 ml of LB broth. On the second day 5 ml of starter culture was inoculated into 500 ml of SOB media (made from Hispanlab readymix, cat. no. H1541) with 1 % glucose and the mixture was incubated at 25 °C until optical density reached 0.5 (approx. five hours). Mixture was then incubated on ice for 15 minutes and centrifuged for 10 min at 4 °C (1200 x g). Supernatant was discarded and pellet was resuspended into 90 ml of ice cold TB (transformation buffer: 10 mM HEPES pH 6.7, 15 mM

$CaCl_2$. 55 mM $MnCl_2$, 250 mM KCl) and split into two 50 ml tubes. After 15 minute incubation on ice, the tubes were centrifuged for 10 min at 4 °C (1200 x g). Supernatants were discarded and pellets were resuspended and combined into 18.6 ml of TB. 1.4 ml of DMSO (dimethyl sulfoxide, Sigma, cat. no D2438) was added drop by drop. After 10 minute incubation the solution was aliquoted into 200 µl portions and snap frozen in liquid nitrogen. Cells were stored at -80 °C for further use.

3.5 Transfecting Cells

A tube of frozen NIH/3T3 cells was thawed in 37 °C water bath for 1 minute. Cell suspension was pipetted into 10 ml of warm growing media which was then centrifuged for 3 min (750 x g). Supernatant was discarded and the cell pellet was resuspended into 10 ml of warm media and transferred into a 10 cm diameter cell culture dish. The media used was prepared by adding 50 ml of FBS, 5 ml of Penicillin-Streptomycin (10,000 U/ml) and 5 ml of GlutaMAX™ (Gibco, cat. numbers 10270106, 15140122 and 35050061) into 500 ml of DMEM with 4.5 g/L glucose, without L-glutamine (Lonza, cat. no 12-614F). Cells were split once before seeding into 35 mm plates. For each plate 100k cells were transferred and transfected with the plasmid the next day using jetPRIME (Polypus transfection, cat. no 114-15) transfection reagent according to the manufacturer's instructions. In addition to the two plates for IRES plasmid (one for the TSA treatment), cells was transfected with H2B-GFP, GFP-mCherry and H2B-GFP with soluble mCherry, one 35 mm plate for each construct. TSA treatment was done by adding TSA (Sigma, cat. no T8552) at a final concentration of 200 ng/ml for 24 hours.

3.6 Imaging

On the third day FLIM was measured with Leica SP5 MP using 860 nm excitation. 128x128 pixels images were acquired and lifetime was measured using PicoQuant SymPhoTime software. Statistical analysis was performed by comparing variances of the sample sets with F-test and then using t-test for uneven variances. Microsoft Excel was used as a software.

4 Results

4.1 Cloning Results

Success of each cloning step was ensured with sequencing done by an external genomics company Macrogen. Samples to be sequenced were chosen by test digestion results. Figure 8 displays some examples of gel images taken after test digestions. Enzymes used are described in methods and materials chapter.

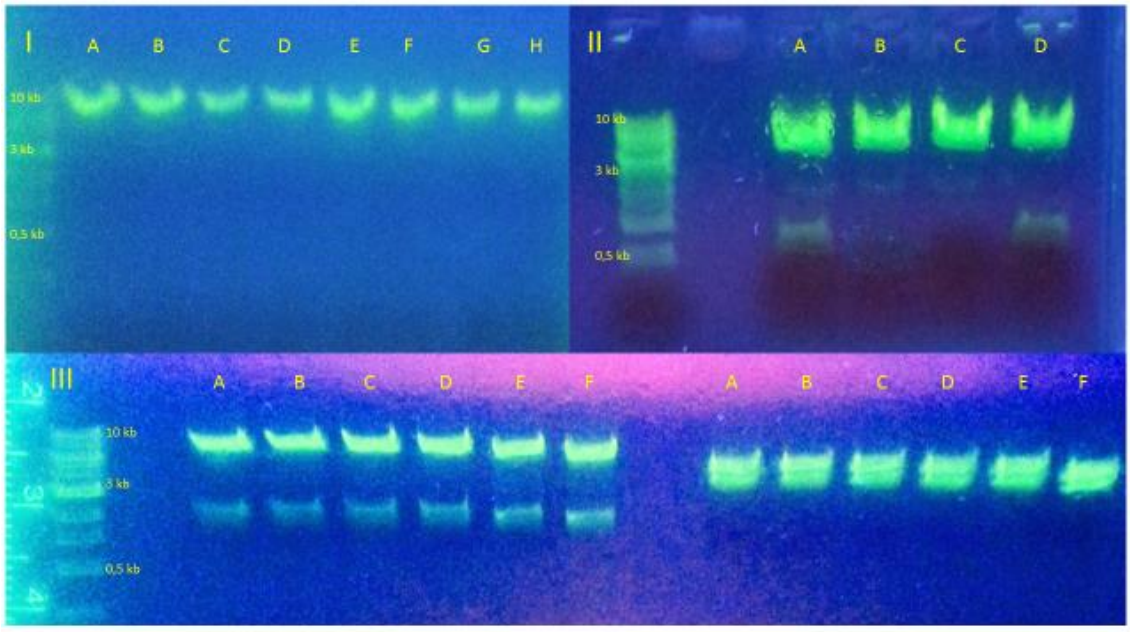

Figure 8. Test digestions: I) an example of unsuccessful cloning of H2B into IRES vector, II) successfull cloning of H2B-mCherry, clones A & D are correct, III) test digestion of the final product with two pairs of restriction enzymes, all six clones are correct.

In the first image a band representing H2B insert should be seen (386 bp). However, no cutting of the plasmid is noticeable, only the vector is visible. This indicates that the cloning was not successful. The problem later turned out to be dam-methylation, which was resolved by using a different competent *E.coli* genotype for transformations.

Second image shows four replicate minipreps done from H2B-mCherry plasmid. Samples A and D can be assumed correct since they have correct size bands representing H2B insert.

Final product in the third image was digested with two pairs of restriction enzymes to ensure that it contains both H2B-mCherry and EGFP-H2B. Predicted band sizes with the first pair of enzymes were ~1100 bp representing H2B-mCherry, and with the second enzyme pair ~2400 bp representing H2B-mCherry-IRES-EGFP. Thus the result can be interpreted as positive for all six replicates.

4.2 Imaging Results

Five cell nuclei of each condition were imaged and the FLIM signal measured and analyzed. Measured data is presented in detail in appendix 2. A representative sample of each are shown in the figure 9. Color of a pixel depends of the lifetime of a donor (GFP) fluorescence, red being the longest and blue the shortest. The shorter the distance between GFP and mCherry, the shorter the lifetime should be.

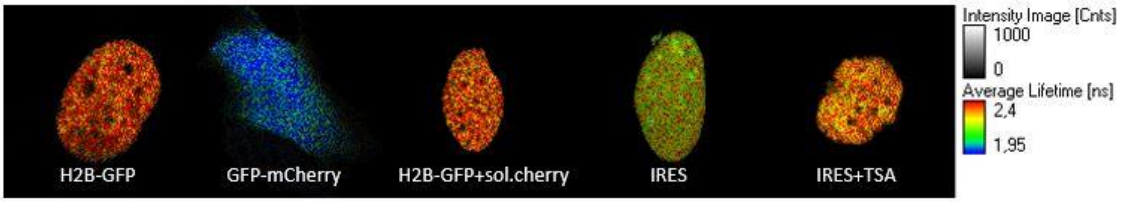

Figure 9. Sample cell nucleus images for each conditions. Colours represent the lifetime of GFP.

From the cell images it can be seen that the difference in color between H2B-GFP (no FRET) and GFP-mCherry (“full” FRET) is significant. This indicates that at least the FLIM-FRET procedure was working well. Not much difference is seen between H2B-GFP and H2B-GFP with soluble mCherry. This was to some extent an expected result and not that important in this experiment.

The most interesting pair of conditions to compare are IRES and IRES+TSA. As described, TSA should cause chromatin to decondense and thus the FRET signal should be noticeably decreased. In the example images of cell nuclei the difference seems to be observable as the IRES nucleus is clearly green and the IRES+TSA nucleus more red and yellow.

Statistical analysis was made from the imaging results as well. Figure 10 shows the mean values of the GFP fluorescence lifetimes for five replicates measured for each condition.

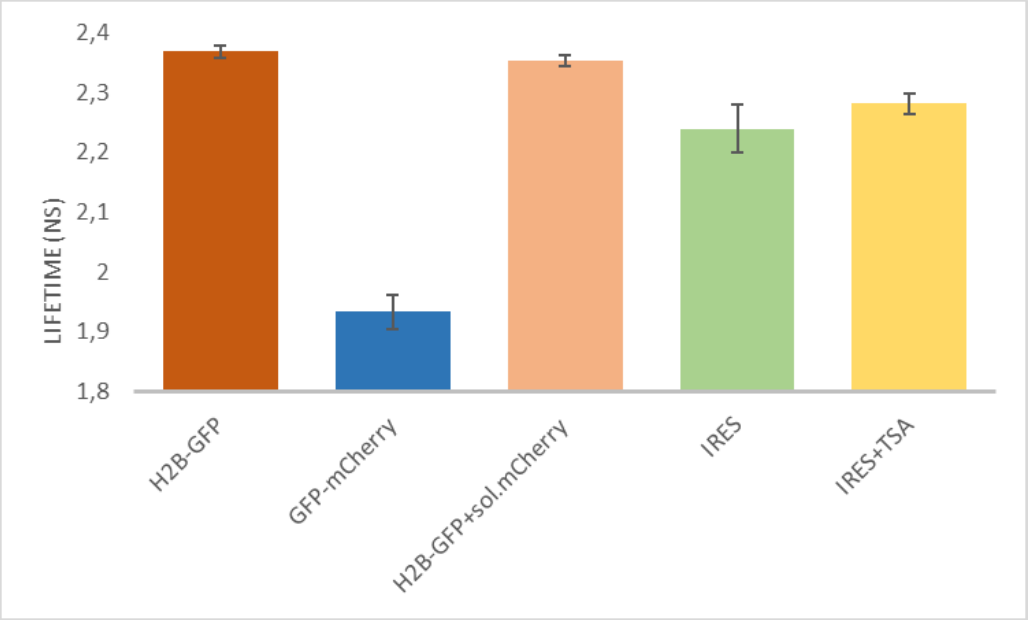

Figure 10. Mean values of lifetimes measured. Error bars represent standard deviation. N=5.

The graph confirms that the H2B-GFP and GFP-mCherry give indeed very unequal signals. However, the difference between IRES and IRES+TSA signals in here is not as distinct as in cell images. To compare them statistically, a t-test for unequal variances was performed, and it showed no statistical difference with P-value 0.05. IRES cells measured had relatively high variance and with a larger sample size the difference could be more significant.

5 Conclusions

The aim of this thesis project was to develop an *in vivo* tool which would allow the level of chromatin condensation to be quantified. Steps to achieve this goal included designing the plasmid, cloning the pieces to the IRES vector, transfecting cells with the plasmid and measuring as well as analyzing data with FLIM-FRET technique.

Plasmid designing and cloning was by far the most time and labor consuming part of the process. Some obstacles had to be traversed during the cloning but in the end the outcome was proven to be a success by test digestions and sequencing results.

Microscopy test in living cells was at least partly successful and most likely a bigger sample size would have yielded even more convincing results. This whole method of using tagged H2B with FLIM-FRET is already proved to work in previous studies and there is no obvious reason why it would not work when it's done with a single plasmid instead of expressing GFP-H2B and H2B-mCherry separately. On the contrary the technique described in this paper should lead to more even expression levels between different tags and thus yield better results.

Future experiments will show if the construct proves out to be useful tool or not. In those studies the sample size needs to be considerably bigger. One example of a project where this tool could be used could include creating a stable cell line where a gene of some key component of DNA damage response is knocked out. Then DNA damage can be induced with for example chemicals or laser light, and the chromatin compaction could be compared between knock-out cell line and wild type to see if the knocked-out protein has any effect.

Overall, as a thesis project, this whole process was very multifaceted and educational. It enabled previously learned skills to be used, but also required plenty of new theory and techniques to be studied. With more time it would have been interesting to perform more testing with the plasmid, but most likely it will anyway be a useful tool in some future studies.

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Plasmid Frames Used for Cloning

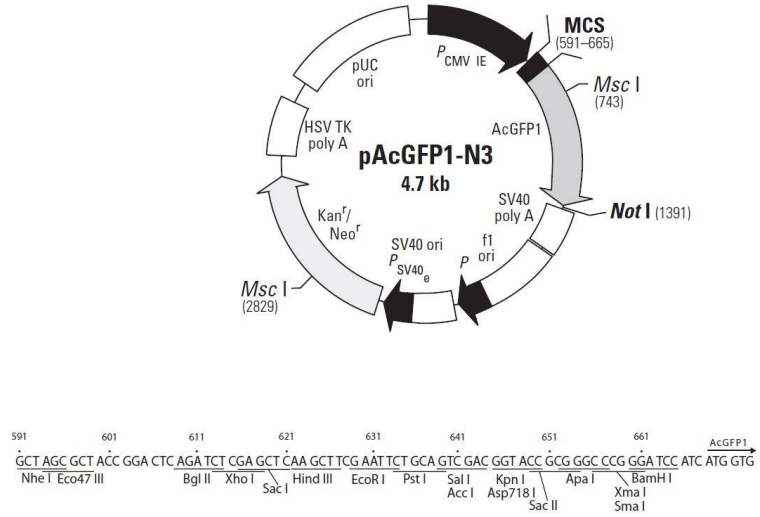

Figure 1. Diagram of the pAcGFP1-N3 frame and the multiple cloning site.

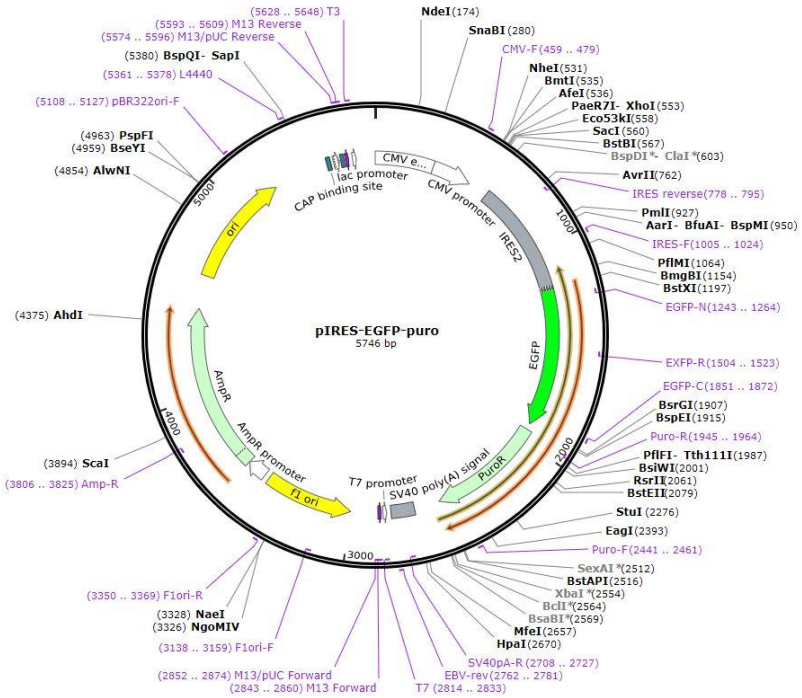

Figure 2. Diagram of the pIRES-EGFP-puro plasmid with restriction sites.

FLIM-FRET Data

Table 1. Measured lifetimes (ns).

	H2B-GFP	GFP-mCherry	H2B-GFP+sol.mCherry	IRES	IRES+TSA
1	2,378	1,894	2,35	2,187	2,276
2	2,351	1,977	2,354	2,255	2,291
3	2,369	1,917	2,367	2,196	2,297
4	2,364	1,93	2,339	2,272	2,25
5	2,376	1,949	2,351	2,283	2,289
mean	2,3676	1,9334	2,3522	2,2386	2,2806
stdev	0,009687	0,028203546	0,008975522	0,039580803	0,016764248