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ÚSTAV FYZIKÁLNÍ A SPOTŘEBNÍ CHEMIE

INFLUENCE OF A COMPOSITION OF HYBRIDIZATION MIXTURES ON FLUORESCENCE INTENSITY DURING THE IN-SITU HYBRIDIZATION

VLIV HYBRIDIZAČNÍCH SMĚSÍ NA INTENZITU FLUORESCENCE PŘI IN SITU HYBRIDIZACI

MASTER'S THESIS
DIPLOMOVÁ PRÁCE

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Master's Thesis Assignment

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Influence of a composition of hybridization mixtures on fluorescence intensity during the in-situ hybridization

Master's Thesis assignment:

1) To collect the theoretical background dealing with in situ hybridization
2) To get acquainted with confocal microscopy and differential scanning calorimetry
3) To design and conduct experiments for characterization of DNA-hybridization mixture system by differential scanning calorimetry and confocal microscopy
4) To evaluate the results and interactions of the hybridization mixture and DNA in relation to the intensity of the fluorescence signal and calorimetric data.

Deadline for Master's Thesis delivery: 17. 5. 2019

Master's Thesis is necessary to deliver to a secretary of institute in the number of copies defined by the dean. This assignment is part of Master's Thesis.

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PROHLÁŠENÍ

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PODĚKOVÁNÍ

ABSTRAKT

Fluorescenční in situ hybridizace (FISH) je široce používaná metoda pro detekci určité sekvence DNA na chromozomech. Cílem práce je porovnání tří různých chemických sloučenin (formamidu, ethylenkarbonátu a sodných kationtů) používaných ve směsích pro *in situ* hybridizaci. Složení těchto směsí ovlivňuje renaturaci DNA a je důležité porovnat jejich fyzikální vlastnosti. Práce je rozdělena do dvou hlavních částí. První část se zabývá otázkou termodynamických parametrů používaných pro experimenty FISH, jako je teplota tání, entalpie přechodu DNA ze dvoušroubovice na vlákno nám dává přehled o energii potřebné k tomto přechodu a interakcích mezi bázemi a každou složkou směsi. Kromě toho hodnoty entropie určují poř uvnitř směsi - systém DNA. Druhá část porovnává intenzitu fluorescenčního signálu při optimalizovaných teplotách tání sondy použité pro *in situ* hybridizaci. Jako sonda byla použita sub-telomerní repetice X43.1, která je umístěna na Y chromozomu rostlinného modelového organismu *Silene latifolia*. Směs obsahující formamid má nejlepší výkon při delším postupu hybridizace, zatímco ethylenkarbonát poskytuje vyšší intenzitu signálu, a proto je vhodnější pro rychlé FISH protokoly.

KLÍČOVÁ SLOVA

Fluorescencční *in situ* hybridizace, průmerná intenzita fluorescence, hybridizační směsi, thermochemie, teplota tání, *Silene latifolia*

ABSTRACT

Fluorescence *in situ* hybridization (FISH) is widely used method for detection of certain DNA sequence on the chromosomes. The goal of this thesis is a comparison of three different chemical compounds (formamide, ethylene carbonate and sodium cations) used in mixtures for *in situ* hybridization. All three mentioned compoundsi nfluence renaturation of DNA and it is interesting to compare their physical properties. This thesis is split into two major parts. First part deals with question of thermodynamic parameters used for FISH experiments, such as melting temperature. Determining enthalpy of helix-coil transition give us insight about newly established bounds between bases and each mixture composition. In addition, entropy values determine the order inside mixture – DNA system. Second part compares optimized probe melting temperatures used for *in situ* hybridization and obtained mean fluorescence signal intensity. As a probe was used sub-telomeric X43.1 repeat, which is located on the Y-chromosome of plant model organism *Silene latifolia*. The mixture containing formamide has the best performance during longer hybridization procedure, whereas ethylene carbonate yielded higher signal intensity and therefore it is more suitable for fast FISH protocols.

KEYWORDS

Fluorescence *in situ* hybridization, mean fluorescence intensity, hybridization mixtures, thermochemistry, melting temperature, *Silene latifolia*

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1   INTRODUCTION

Molecular laboratories studying genes, genomes and whole organisms need to test the presence of certain DNA loci in a sample. As methods are often used southern blot hybridization, PCR and its modification, and fluorescence *in situ* hybridization (FISH). Among others, FISH as only method allow to visualize studied DNA sequence with relatively high precision on chromosome. This method allows. This method allows resolution of single kb (in case of fiber FISH). Process of hybridization is time consuming (24-72 hours) and is influenced by number of variables as denaturation (and renaturation in return) temperature, concentration of ions in solvent, denaturing agent in hybridization mixture or by and type of fluorochrome used for DNA probe labeling. Classical hybridization mixture is using formamide, SSC, dextran sulfate and ssDNA to bind probe on specific genome region. In recent years was described several new protocols, which are avoiding usage of formamide and replacing it with substances that are less geno-toxic or faster in process of hybridization than mixture containing formamide[1], [2].

2 THEORETICAL PART

2.1 DNA

2.1.1 Introduction

Basic structure was discovered by Watson and Crick in 1952. DNA is considered largest biological polymer [3]. Nucleic acids, polymers of several building blocks called nucleotides, which store and transfer all genetic information in living cells and organisms. DNA is structure, which consists of three major parts: nitrogen containing base, 5-carbon sugar deoxyribose and phosphoric acid. The polymer is connected by phosphodiester bonds between deoxyriboses moieties of the nucleotides, which are only covalent lineages in the polymer [4].

2.1.2 Structure

DNA consists of two strands that are wrapped clockwise around central axis to form shape of double helix. Strands are held together by hydrogen bonds formed between bases, which can be divided into two groups based on their chemical structure. Template for base structure is molecule of purine or pyrimidine. Purine based bases are Adenine (A) and Guanine (G). Thymine (T) and Cytosine (C) are derived from pyrimidine cycle as shown in Figure 1 [4].
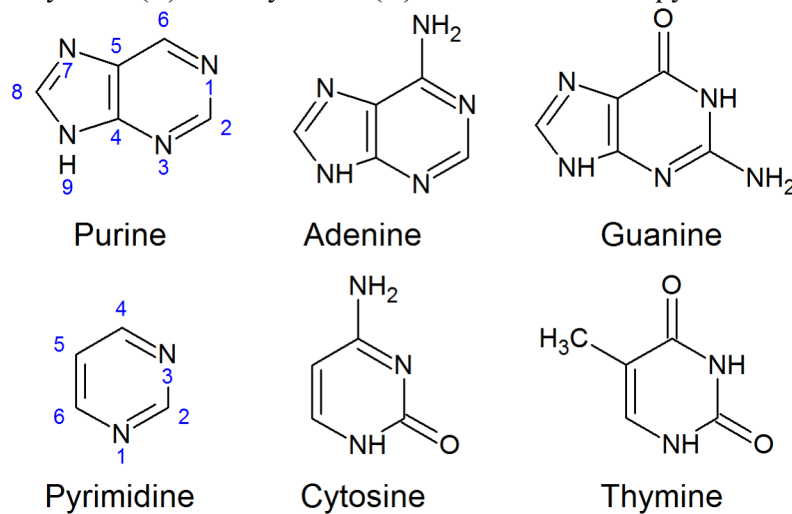
Figure 1: DNA bases

Nucleotides do not form pairs randomly. Adenine only ties to thymine and cytosine links only with guanine creating complementary pairs. Because of this rule, the same information is present in each half of the double helix [3]. The difference between mentioned pairs are number of hydrogen bonds. Adenin and thymin share two hydrogen bonds, whereas the CG pair shares three. The difference in number of hydrogen bonds has impact on the force that holds DNA strands together, and as such can be observed in experiments regarding melting temperature of DNA [4].

Sugar in form of 2-deoxyribose and phosphoric acid acts as backbone for helical structure. Sugar connects with nucleotides through glycosidic bond between nitrogen and carbon and creates deoxyribonucleoside. Bond is created between carbon with aldehyde group (C-1) and base nitrogen (N-1 for pyrimidine and N9 for purine based bases) providing hydrogen for dehydrogenation during creation of glycoside link. Adding phosphoric acid to nucleoside creates nucleotide. Acid can be esterified to 3' or 5' in the deoxy-ribose molecule [4].

The bases have a tendency to stack upon each other, using its energy of van der Waals interaction of π-stacking. Bases are almost in perpendicular position to long axis of the polymer stacked 0.34 nm apart with diameter of 1.9 nm. This form is referred as DNA B-form and its polynucleotide chain has 10.5 pairs per turn. Because of asymmetrical bonds between the bases and the sugar, the DNA helix is asymmetrical as well creating major and minor groove. Grooves plays a role in interaction between DNA and proteins. Most of cellular DNA can be found in the B-form conformation. Other existing forms of DNA chain are A-form and Z-form. These forms differs form B-form in base pairs form turn. A-form has then broader diameter and Z-form is wrapped anti-clockwise [4].

2.1.3   Stability of polymeric structure

Polynucleotide chain secondary structure is stabilized by four major non-covalent interactions. Hydrogen bonds, hydrophobic effect and van der Waals interaction are localized between DNA bases. Ion interactions are present between phosphoric acid in backbone of polymeric chain and solvent.

Hydrogen bonds are stabilizing secondary structure between individual base pairs and play major role in forming helical shape of DNA polymer. Hydrogen bonds are highly directional and posses energy between 12 to 29 kJ per mol [4].

Hydrophobic effect is generated by hydrophobic molecules in hydrophilic solutions (water), but it is not bond per se and it is considered more as thermodynamic factor. It is described that entropy of system increases when association of molecules takes place. Increase in entropy means increased instability of system. By organization of polymeric structure the solvent looses its order, which then offsets the increased order of polymeric structure.

Van der Waals interactions take place between bases as well and have significant impact on stability of secondary structure of DNA. This action occurs between outer electron clouds present above and below covalent double bond of carbons in purine and pyrimidine cycles. Van der Waals interactions are highly dependent on distance, decreasing in proportion to the sixth power of the separation. The energy of single interaction is not as strong compared to hydrogen bond, but when combined as interaction of complementary surfaces it can reach 40 kJ per mol in optimal conditions [4].

Electrostatic bonds are present between charged groups. In DNA the ionic interactions take place between solvent in form of cation (for expample Na^+) and phosphoric acid acting as anion. Ionic bonds are potentially as strong as hydrogen bonds [4].

Composition of solvent surrounding DNA double helix is an important factor. For example water or small molecules possessing OH group can act as competitor during hydrogen bonds creation and in fact both molecules influence energy of H-bonds between nucleotides in negative matter [5], [6]. Electrostatic bonds are highly affected by concentration of cations in solution such as Mg^+ and Na^+. Van der Waals interactions depends on distance and presence of electron clouds [5].

2.2 Hybridization solutions

Fluorescence *in situ* hybridization is still the method of choice for chromosome studies since its invention. Big improvements were made in field of image acquisition. Confocal microscopy enabled better resolution in two-dimensional space compared to epifluorescent microscopy with additional three-dimensional scans of studied sample.

Fluorophores are constantly evolving in the way of better stability and higher yields of fluorescence signal. These advances enabled us to measure and capture signals much smaller than was previously possible - from repetitive sequences like rDNA and other satellites to single copy genes spanning from 3.1 kb on stretched pachytene chromosomes and amplification of the signal by tyramide [7].

Hybridization solutions were overlooked for long time. Purpose of these solutions is to lower effective melting temperature of DNA double helix and therefore protecting DNA from extensive heat and losing information. They destabilize the secondary structure of the DNA by interfering with its non-covalent interaction described in previous chapter.

2.2.1 Formamide

Formamide is a small molecule capable of insertion between complementary pairs (Figure 2) and divide hydrogen bonds between bases due to creation stronger H-bonds with individual bases. Formamide hybridization techniques are widely used across cytogenetic laboratories. For 30 years it was hybridization agent of choice [1]. Although its widely use, formamide has its drawbacks. Among the most important is genotoxicity and potential teratogenity [27]. Second one is long incubation time and low efficiency of low-copy and single copy hybridization targets.
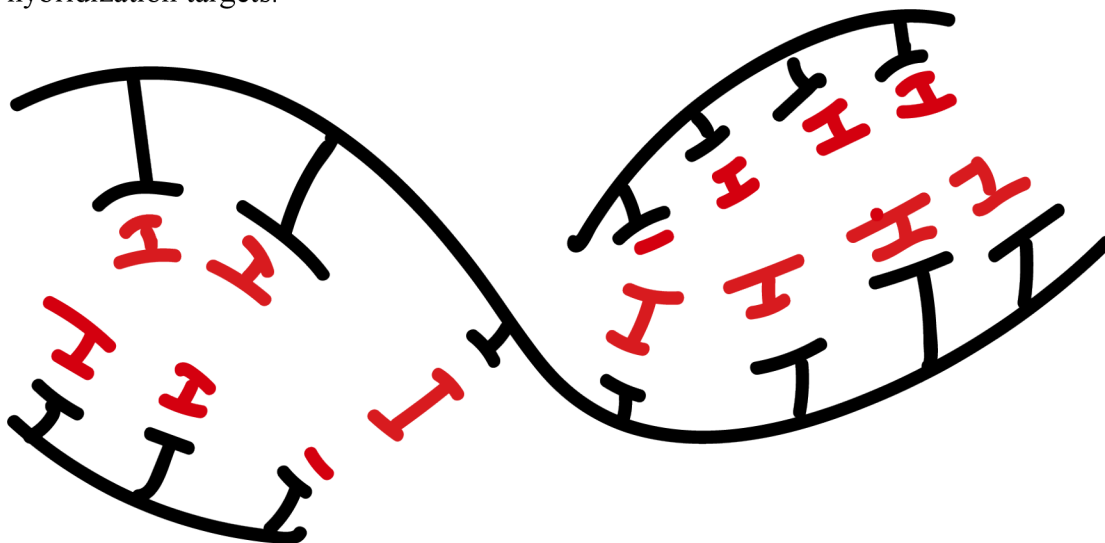

Figure 2: Simplified representation of formamide-DNA insertion and destabilization of hydrogen bonds between coplementary pairs

Formamide decreases melting temperature linearly by 2.4-2.9°C per mole depending on base content, helix conformation and state of hydration. These characteristics of DNA content are showing to be responsible for small deviation from linear pattern. Three hydrogen bonds CG pairs have higher melting temperature than two bonds containing AT pair. .

Denaturing ability of formamide is credited to lower ion-solvating power and better free bases solubility. Fromamide increases hydrophobic character of the solvent, which leads to opening of the core of the double helix, creating space for formamide to disturb hydrogen bonds between bases and favoring the denatured state of DNA polymer [8].

2.2.2 Ethylene carbonate

Ethylene carbonate is heterocyclic organic compound. Its considered to be polar aprotic solvent with significant dipole moment (Table 1.)

Table 1: Dipole moments of relevant substances

Molecule	Formula	Dipole moment
Water	H_2O	1.85
Adenine	$C_5H_5N_5$	2.56
Guanine	CH_2O	6.55
Formamide	CH_3NO	3.73
Ethylene carbonate	$C_3H_4O_3$	4.90

Dipole moment is considered to be important part of π-stacking between DNA bases (van der Waals interaction). The electrostatic changes may occur considering charge fluctuation, partial charges on different parts of heterocyclic molecules, dispersion effect depending on surface area, polarizeability and solvent driven effects. Generally purine bases are more effective in stacking than pyrimidine ones. Important factors in destabilization of DNA are solvophobic and other solvent driven effects. Hydrophobic stacking is one of the main driving forces in stabilization of the DNA double helix. In general, adding organic solvents destabilize the DNA duplex [9]. Hybridization solvents attacking van der Waals interaction instead of hydrogen bonds seems to have some advantages over formamide H-bonds driven denaturation. (Figure 3)
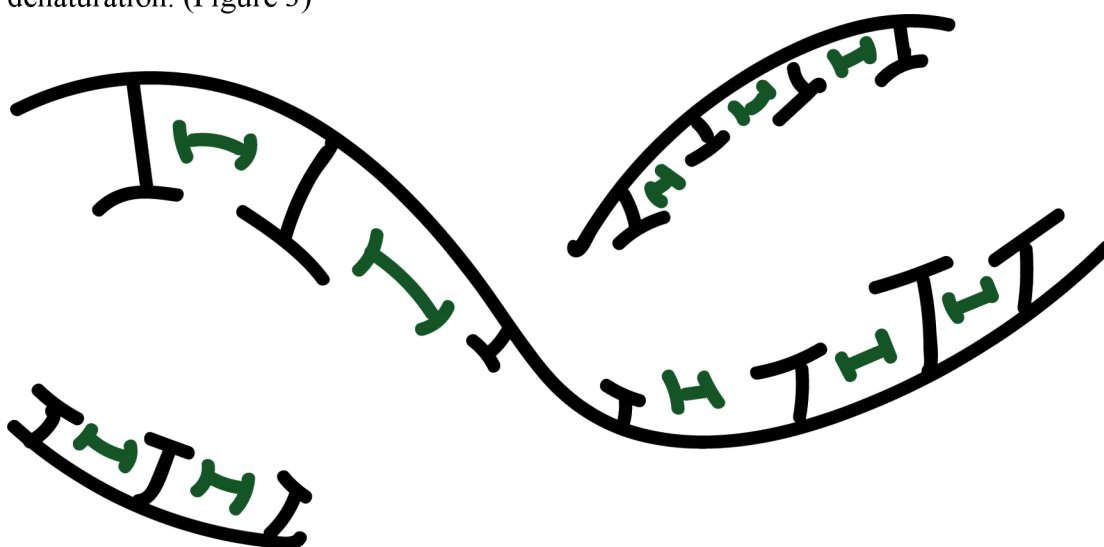

Figure 3: Simplified representation of ethylene carbonate interaction with base stacking

Solvents destabilizing DNA base stacking are characterized by lower miscibility with other parts of hybridization solution. Based on Hansen solubility parameters for DNA, low hydrogen bonding solubility and molar volume, ethylene carbonate has sufficient properties for *in situ* hybridization as replacement for formamide as fast, non-toxic hybridization mixture [1].

2.2.3 Sodium concentration

Influence of cation stabilization effect on DNA duplex and melting temperature was extensively studied in past. Various algorithms were presented in relationship of sodium ion concentration and melting temperature of DNA double helix [10].

The electrostatic free energy change in the helix-coil transition is related to electrostatic repulsion between phosphate charges of two strands. Stability of secondary DNA structure is affected by high local concentration of cations (Na^+, Mg^{2+}) with opposite charge to phosphate groups present in DNA backbone. Counter-ions in the vicinity are covering the fixed charges to the degree in which the systems behave as if the fixed phosphate ions carried reduced charge (Figure 4). The decrease in melting temperatures in environment of low sodium ions is caused by increased electrostatic repulsion of phosphate groups that prefer strands separation [11].

Sodium ions are also present in so called alkaline denaturation, where it's commonly used in conjunction with OH anions. Denaturation of DNA is caused by increased pH. Hydroxide groups are responsible for weakening of hydrogen bonds between nitrogen bases by removing protons from guanine and thymine responsible for creation of hydrogen bonds. Level of denaturation is in direct proportion to NaOH concentration [12].

Figure 4: Simplified representation of Na catioons shielding the phosphate anions in DNA backbone

2.3 Fluorescence *in situ* hybridization (FISH)

Fluorescence *in* situ hybridization is multidisciplinary technique requiring skills in cytogenetics, molecular biology, immunocytochemistry, microscopy and image analysis. *In situ* hybridization is widely used technique utilizing fluorescently labeled nucleotides to visualize complementary sequences in the genome. Using this approach, it is possible to study and provide evidence of rearrangements and changes during evolution, organism development and disease. Hybridization methods have improved our understanding of structure, function, organization, and evolution of genes and the genome. It has enabled the linkage of molecular data about DNA sequence with chromosomal and expression information in the specific tissue and on cellular and sub-cellular level. *In situ* hybridization is common and established technique, with countless laboratories publishing using this method [13].

Technology was initially developed for mammalian chromosome research as a tool for physical mapping, helping to visualize genes within chromosomes and than transferred to plant species by Schwarzer et al., Yamamoto and Murai. First hybridizations in plants were performed with rDNA probes, such as 18S and 26S, visualizing these sequences on chromosomes of *Arabidopsis thaliana, Glycine max* and *Brassica* [14]. This allowed later studies in agronomically important species, such as maize, corn, sugar cane and others. Dioecious plants, such as *Rumex, Silene* and *Humulus* were also subjects of fluorescent *in situ* hybridization because of the properties of their sex chromosomes.

2.3.1 Chromosome preparation

Preparation of high-quality chromosomes is crucial step in plant FISH experiments. Cellular components such as cell wall, cytoplasm and secondary metabolites are increasing the background and decreasing the intensity of fluorescence signal. Chromosomes should be prepared without overlaps and changes in their morphology for easy recognition and count.

In plant cytogenetics, the material for chromosome preparation usually comes from hairy roots, root tips and young floral meristems.

The first step of chromosome preparation is cell synchronization and accumulation of synchronized cells in methaphase. Number of chemical is used for cell synchronization, such as treatment with hydroxyurea, mimosine, aphidicolin and anti-tubulin substances like the alkaloid colchicine, oryzaline, nitrous oxide gas amiprosphos-methyl. Most methods are based on arresting cells in the G1/S phase. Treatment generally takes longer than the length of cell cycle to secure homogeneity of nuclear content. Time for synchronization is species-dependent. Longer treatment intervals leads to excessive cell death events, nutrient starvation and chromosome breakages that can be followed by irreversible sister chromatid exchanges. By removing synchronization agents, cells are resuming normal cell cycle [15].

Anti-tubulin drugs mentioned in paragraph above accomplish the accumulation of chromosomes in metaphase.

To avoid so-called ball metaphases, cold treatment is recommended. This leads to high mitotic index without strong overlapping of chromosomes that are often present when using the anti-tubulin agents such as colchicine.

Release of chromosomes from cells is basic step for *in situ* hybridization. Removing cell envelope is easier for animal cells than for plant cells that contain additional cell wall that needs to be disrupted by enzymes, usually containing cellulose and pectin hydrolases among others. This step needs calibration of enzymatic mixture composition and duration of enzyme treatment to achieve chromosomes, which are free of cell wall, secondary metabolites and especially cytoplasm [15].
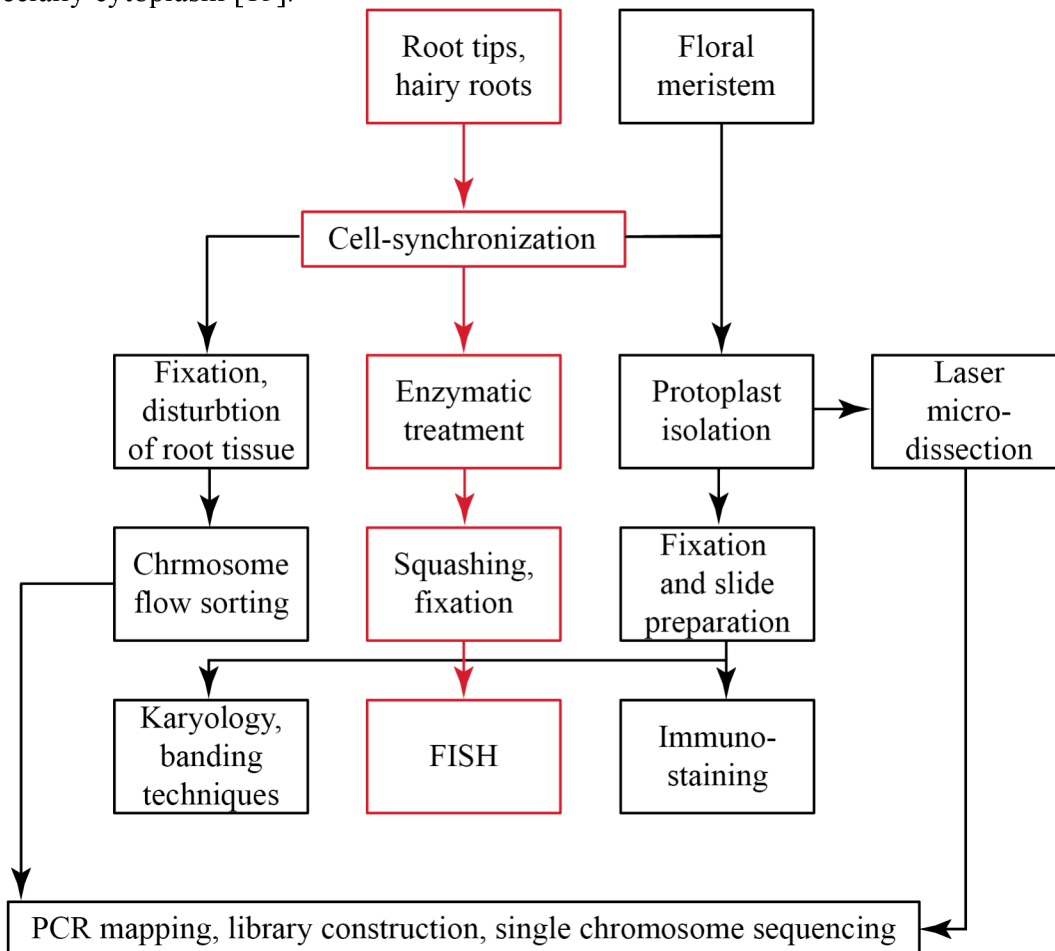

Figure 5: Workflow diagram for chromosome preparation and manipulation in plant cytogenetics

2.3.2   DNA fluorescent labels

Several methods of non-radioactive labeling used in *in situ* hybridization became available over last decades. The most common methods for DNA and RNA labeling are based on binding the fluorescence active compound to the uridine base, which is used in almost all protocols except for probes with high CG content. These labels are dependent on fluorescently stained C and G bases to render sufficient signal [13].

Incorporation data of labeling and signal detection are not definitive, but the 100% replacement of nucleotides is not the aim of labeling. Steric changes in the probe affect probe-binding ability and high incorporation of labeled nucleotide, which is undesirable. To address this issue, chains of four to twenty aliphatic carbon compounds are usually used to connect the fluorescent/hapten-group and nucleotide to limit these effects [13].

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Fluorescent detection systems are usually favored for DNA, RNA and proteins that are well-defined locations on chromosomes. Signal from these sequences is characteristic and easy to determine signal from noise. Fluorophores are now able to cover more than visible spectrum of light. Even though colorimetric detection provides stronger signal, it is used only for RNA *in situ* hybridization, because of limitation in spatial specificity [13].

Two approaches can be used when creating the non-radioactive probes for *in situ* hybridization – indirect and direct [13].

First approach is using reporter molecules known as haptens. Most common and commercially available haptens are based on biotin, digoxigenin and dinitrophenol [14]. Biotin has high affinity to avidin. Complex biotin-avidin is available for fluorescent detection of hybridization and can be incorporated as labelled nucleotides into DNA via nick translation, random primer labeling or polymerase chain reaction (PCR). Detection of probe hybridization sites, avidin or anti-hapten antibodies are linked to fluorescent moieties or enzymes, which precipitate chromogenic substrates [13].

Fluorescein can be used in both approaches. As well as being direct label, it has good antigenicity and high affinity for its antibody.

Second path to obtain labelled probe for *in situ* hybridization is direct conjugation of fluorophore and nucleotide. This approach is faster and simpler then using antibodies and often produces less non-specific signal than non-direct probes [13].

2.4 Thermochemistry

The study of transferred energy as heat during chemical reaction is called thermochemistry, which is part of thermodynamics. Chemical reactions are accompanied by exchange of energy between system compounds. To measure these changes, calorimetry can by used to measure amount of energy consumed or expelled as heat. Reaction of system that releases energy as heat into its surrounding is called exothermic and opposite reaction is called endothermic [16].

2.4.1 Heat capacity

Heat capacity (C) is important physical quantity characterizing behaviour of substances under heat gradient. It can described as heat absorbed by system. Experimentally it can be measured as a change in system temperature. Substance capable of absorbing great amount of heat has a high value of heat capacity, vice versa systems prevalent to temperature change are considered having low heat capacity. This phenomenon can be illustrated by equation (1:

$$\mathrm{d}Q = C \cdot \mathrm{d}T \tag{1}$$

Heat capacity is dependent on more factors: system size, heat transfer conditions and state of matter.

System size is usually related to 1 mol – molar heat capacity (C_m) mass unit – specific heat capacity (C_s).

Heat transfer conditions are related to constant volume and constant pressure. These are described by equations (2 and(3:

$$C_V = \frac{\mathrm{d}Q_V}{\mathrm{d}T} = \left(\frac{\partial U}{\partial T} \right)_V \tag{2}$$

$$C_p = \frac{dQ_p}{dT} = \left(\frac{\partial H}{\partial T} \right)_p \tag{3}$$

If the condition is described by constant pressure the system is doing work by changing the volume. Operating under constant volume causes all the heat to transfer into change of temperature.

State of matter defines movement of molecules. Values of substance's $C_{m,\,p}$ is rising from solid state through liquid to gas phase. Heat capacity is not defined for phase transition [17].

2.4.2 Enthalpy

Enthalpy is described as sum of systems internal energy. It's derived from three state functions: internal energy, pressure and volume.

$$\mathrm{d}H = \mathrm{d}U + p \cdot \mathrm{d}V \tag{4}$$

Like U, p and V, enthalpy is a function of state, because it is defined in terms of quantities that are all function of state. As for any state function, the change in enthalpy is independent on path between initial and final state of the system.

Mixing process is rarely athermal. Energy of cohesion forces changes, which is transformed into heat exchange and change of enthalpy.

Enthalpy change can be measured calorimetrically by recording the temperature change, which is caused by changes in physical or chemical state of system that is considered isobaric or isochoric. The most sophisticated way to measure enthalpy changes, is to use differential scanning calorimeter (DSC). From DSC the enthalpy can be calculated form heat capacity by equation:

$$\Delta H = \int_{T_1}^{T_2} C_p \, dT \qquad (5)$$

[18].

2.4.3 Entropy

Entropy is part of second thermodynamic law and can be presented as level of molecular disorder or randomness. As system becomes less organized, the position of molecules less predictive and entropy increases. Entropy is lowest in solid phase and highest in gas phase. The entropy of a system can be viewed as number possible microscopic state of molecular configuration. The total number of microscopic states of that system, called thermodynamic probability W is expressed by the Boltzman equation:

$$S = k \cdot \ln W \qquad (6)$$

To visualize of systems of polymer and solvent, such as DNA and hybridization solution, is done by introduction of lattice model (Figure 6). Open spaces can be filled with molecules of solvent or segments of polymeric chain and the lattice is representation of liquid phase [19]
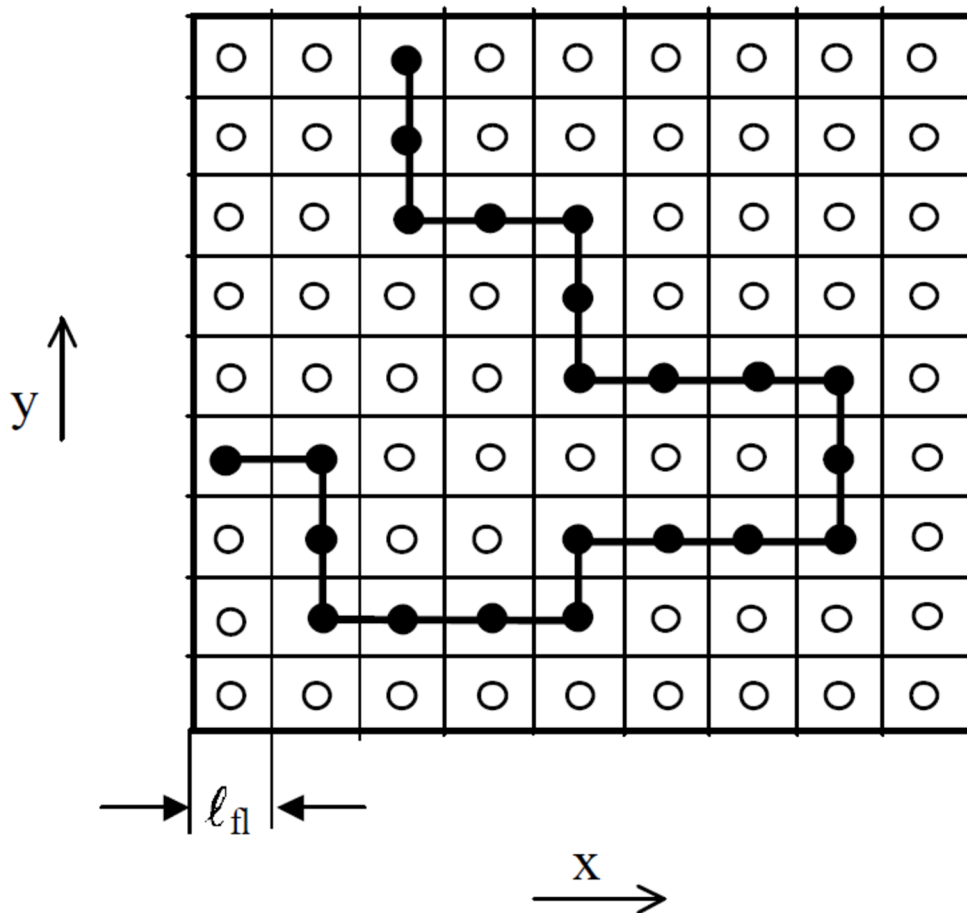
Figure 6: Lattice model for polymer-solvent mixture

Entropy can be obtained form DSC by equation [20]:

$$\Delta S(T) = \int_{T_1}^{T_2} \frac{\Delta C_p}{T} dT \qquad (7)$$

2.4.4 Melting temperature

The melting temperature T_M in DNA thermodynamic studies refers to denaturation of DNA secondary structure (usually B-form), where 50% of DNA exists as single strand DNA (ssDNA) and the other 50% in double stranded form (Figure 7). It is dependent on DNA base content. The melting temperature is important characteristic to many techniques in molecular biology, such as polymerase chain reaction (PCR), hybridization or biomolecules stability studies. Techniques for investigating the half-way point of denaturation of DNA, protein or different biopolymer are usually spectrometric or calorimetric.
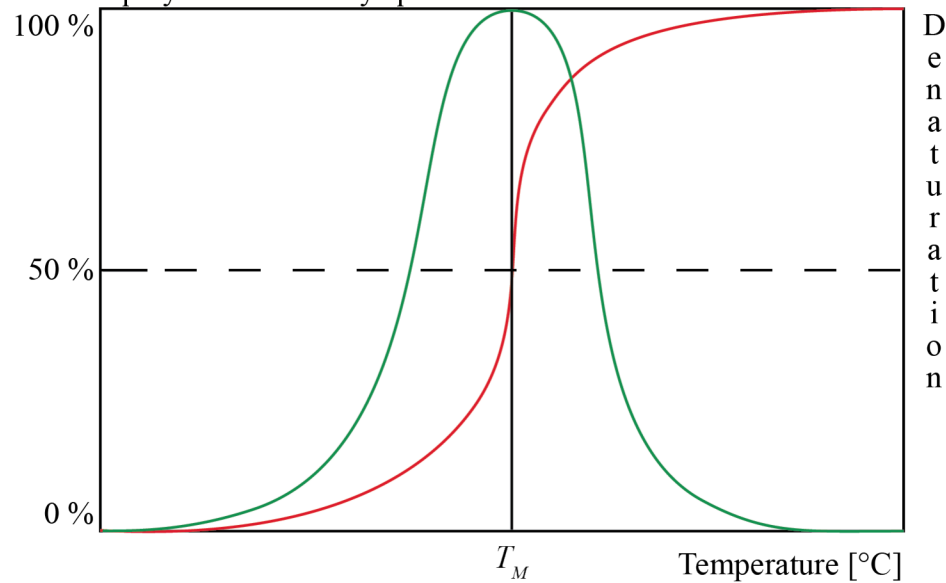

Figure 7: Melting curve

2.5 Differential scanning calorimetry (DSC)

Chemical reaction and many physical transitions are accompanied with change of the temperature by consumption or generation of heat in systems undergoing changes in their structure. Calorimetry is the study of heat transfer during processes generating change of chemical or physical properties of the measured sample. The "differential" refers to fact, that the behavior of sample is compared to that of a reference material that is measured in same timeframe, but does not undergo the changes in chemical or physical structure during the analysis. The word "scanning" refers to the fact that the temperatures of the sample and reference material are increased, or scanned during the analysis [18]. Objective of calorimetry is to measure that heat exchange. In addition DSC is used to measure heat flow rates and characteristic temperature of reaction or transition as well.

A DSC consists of two measuring units that are heated electrically at constant rate. A computer controls the power supply, which maintains the same temperature in both compartments. Temperature is measured and calculated by equation:

$$T = T_0 + \alpha t \qquad (8)$$

One of advantages of DSC is relatively small amount of sample. Approximately 0.5 mg is sufficient for measuring enthalpy changes, which is big step-up compared to standard calorimeters that requires several grams of material. DSC is used in chemical industry to characterized polymers, their structural integrity, stability, and nanoscale organization. Technique can be used to investigate stability of proteins, nucleic acids and membranes and can detect changes in conformation of these structures in which a large number of non-covalent interaction are broken in result of denaturation [18].
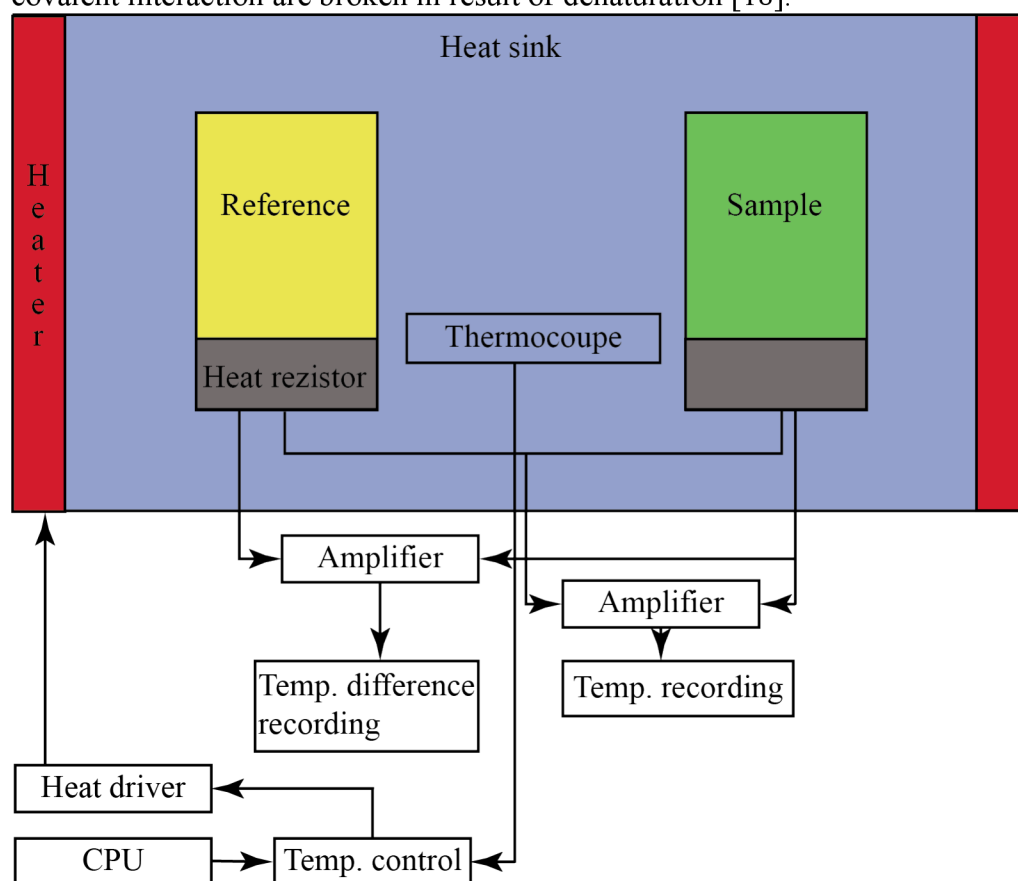

Figure 8: Scheme of differential scanning calorimetry

2.6   Confocal microscopy

Laser scanning confocal microscopy (LSCM) is modern optical imaging technique utilizing spatial pinhole to increase optical resolution by projecting signal only from focal plane. This blocks out of focus signal that is usually cause of lower image resolution or bluring [21].

Setups, such as epi-fluorescence microscopy also known as diffraction limited have limit resolution of 200-300 nm, which was derived by Ernst Abbe as half of shortest visible light (450 nm) acting as excitation signal. Epi-fluorescent microscope acquires signal from all fluorescent molecules in single image. LSCM is not affected by Abbe's limit, because image is acquired from single fluorescent molecule at the time [22].

LSCM technique is extensively utilized in cell biology, genetics, microbiology, medicine and developmental biology [23]. To image the surface is necessary to laterally scan whole plane. Scanning can be managed by number of different techniques, such as multiple scanning disk or electronically controlled mirrors. Scanning speed also referred as laser dwell time sets acquisition of sample [22].
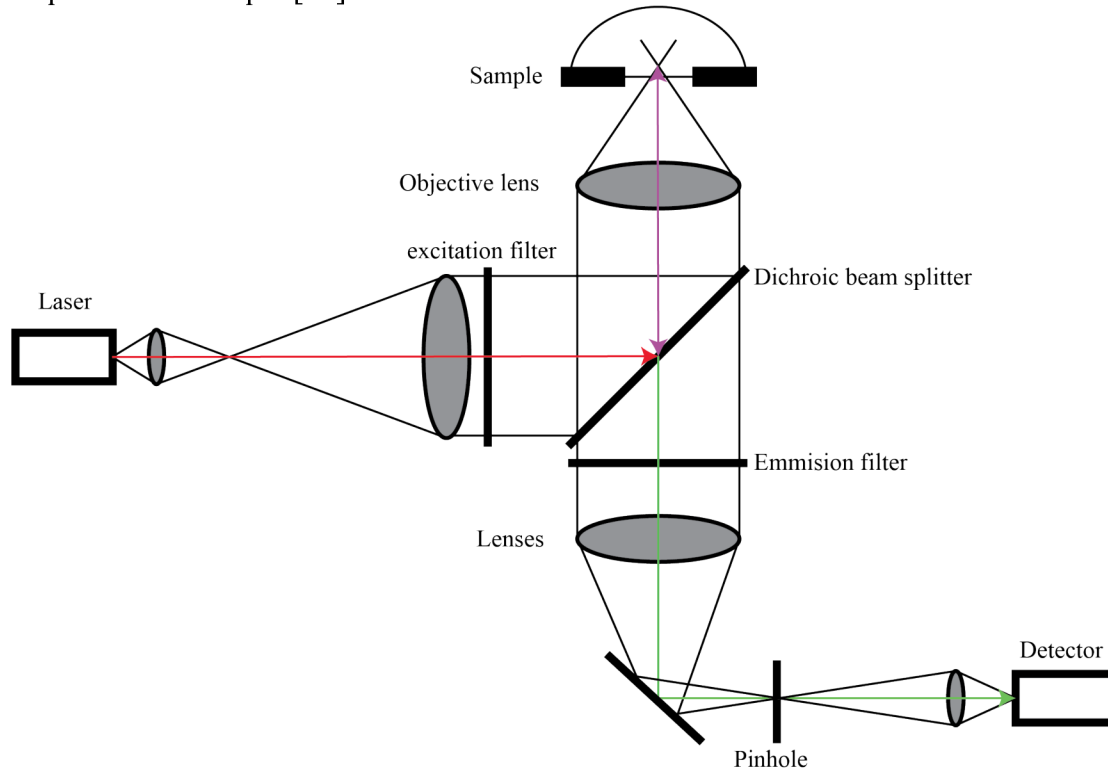

Figure 9: Scheme of confocal microscopy

3   STATE OF CURRENT LITERATURE

Golczyk et al. recently published article describing the use of ethylene carbonate as denaturing agen of fluorescence in *in* situ hybridization. FISH experiment requires experimentally determined heat denaturation of a probe and a sample at high temperatures and at least overnight hybridization. Nevertheless, high temperatures may damage chromosomal preparations and often lead toheat-deterioration of chromosomal structures. Beside, most used FISH protocolas involve toxic formamide

Inhalation and/or skin contact with formamide can cause respiratory tract irritation, headache and nausea, and long-term damage to internal organs, as well potential embryotoxic and teratogenic effects during pregnancy

Formamide as organic solvent has been widely used as a standard component of hybridization solutions to lower the DNA melting temperature by reducing thermal stability of the DNA double helix. Formamide lowers the temperature but also slows down the rate of renaturation, considerably prolonging the time of hybridization.

Hybridization solution that contains 40-50% of formaminde allows denaturation of chromosomal DNA at 70-80 °C for several minutes and to run subsequently a relatively stringent – at least overnight hybridization at 37-42 °C. Several minutes at 70-80 °C may sitll cause to deterring of the chromosome.

The EC-FISH technique mentioned by author doesn't use toxic fromamide and requires no treatment for repetitive sequences of chromosomes, no chromosome/probe heat denaturation, and can be shortened to a 1-day procedure wtih 3 h of hybridization at 46 °C or 50 °C if required.

The EC-FISH tended to preserve well chromosome structural details, e.g., DAPI-positive bands, thus facilitating simultaneous FISH mapping and chromosome banding on the same slide [24].

Sinigaglia et al. published article, in which the author substitude classic formamide with urea substituting classic denaturing solution containig 50% Formaminde for the soft bodied hydrozoan medusa *Clytia hemisphaerica*. Formamide hybridization was causing extensive deterioration of morphology and tissue texture, which compromised observation and interpretation of results.

In their work, 50% formamide mixture was substituted with equal volume of 8M urea solution in the hybridization buffer. New protocol has yielded better morphologies and tissue consistency, but also improved the resolution of the signal, allowing more precise localization of gene expression and reducing non-specific staining associated with problematic areas.

In all cases, formamide and urea variants gave comparable expression patterns, demonstrating that 4 M urea could efficiently substitute for the 50% formamide during hybridization steps. The urea-based protocol produced sharper staining patterns compared to the formamide one, particularly in the case of isolated cells, where single positive cells could be more easily distinguished within the tissues.

Overall, substitution of formamide by urea during in situ hybridization offers a safer alternative, potentially of widespread use in research [25].

Jang et al. published an article regarding the formamide free hybridization solution for genome *in situ* hybridization (GISH). In their work the effective melting temperature of DNA is controlled by sodium concentration. Typically hybridization mixes contain 50% formamide, which allows hybridization at 37-42°C with relatively high stringency. In absence of formamide, hybridization time and temperature are the most sensitive parameters. Additional factors, such as degree of DNA condensation and pH play their role as well. The probe was denatured at 72°C and was hybridized at 37°C and washed at 42°C.

The current ff-GISH protocol does not require prolonged hybridization times. Hybridization efficiency after 12 hours does not significantly increases after 24 hours [2].

4 PRACTICAL PART

4.1 Materials and methods

4.1.1 Model organism

As model organism was chosen diocious plant *Silene latifolia.* This plant possesses 22 autosomal chromosomes and two sex chromosomes XY. The intensity of fluorescence signal was measured on Y chromosome, characteristic by his size as largest chromosome in *Silene latifola's* genome.

4.1.2 DNA sequence for calorimetry

As sequence for calorimetry the 125 bp was picked. This length is average for NICK translation products (after labelling). The sequence is balanced with CG content is 42.7%.

Primary sequence:
AGAGTAGTACTAGGATGGGTGACCTCCTGGGAAGGCCTCGTGTTGCACCCCTTTT
TTTCTTTTTTTCGTTTTTTTTCACGGTACTTTGCCTCCAAATTCTCTTGGTTTAATGAT
TTAAACGCGTAG

Concentration for DSC was 1mg/ml [26]

4.1.3 Preparation of chromosomes for FISH

Chromosomes were obtained from root tip meristems by following procedure:

1. Take seedling from freezer (stored at -20°C) and let them temper

2. Take 6-10 seedlings and move them into 1% carmine acid solution for better contrast of meristem tissue for 1.5 hours

3. Prepare 1mM citrate buffer from stock solution (concentration 10 mM) and adjust pH to 4.6

4. Place beakers with citrate buffer from step 3 and distilled water to ice container and approximately 2 μl of distilled water into hourglass.

5. Pull out seedlings from carmine acid into prepared hourglass

6. Using pincers and scalpel to get rid of tissue that was not stained by acetokarmine solution

7. Wash 2 times for 5 minutes in distilled water

8. Wash 2 time for 5 minutes in 1mM citrate buffer

9. Move root tips from citrate buffer into enzymatic mixture (1% cellulase R10 – Duchefa, 1% cellulase – Sigma Aldrich, 1% pektinolyase – Sigma Aldrich, 1% cytohelicase – Sigma Aldich, 0.5% pectinase) and let incubate at 37°C for 24 – 28 minutes (can be adjusted on based on enzyme activity)

10. After incubation try squash one root tip under cover glass to ensure quality of batch. According to result of squash, repeat step 9. for additional time or move to step 11.

11. After incubation move root tips into hourglass with citrate buffer and keep it on ice.

12. Put root tip on microscopic slide into 12-14 µl of 45% or 60% acetic acid

13. Squash root tip under cover glass using wooden sharp tip (toothpick)

14. Observe chromosome under light microscope (20-40x objectives are sufficient)

15. Remove excessive cytoplasm by heating up the microscopic glass over ethanol burner

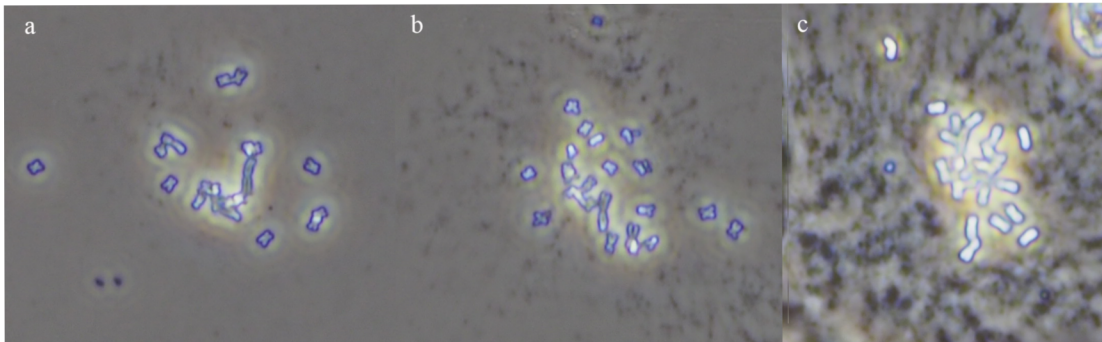16. Mark your microscopic glass according to quality of squash

Figure 10: A comparison of S. latifolia chromosome with various level of cytoplasm. a) chromosomes almost without a cytoplasm b) chromosomes slightly covered by cytoplasm c) chromosomes under cytolasm

17. Mark position of cover glass with diamond tip

18. Put microscopic glass into liquid nitrogen (leave it in until nitrogen stops boiling)

19. Remove cover glass with razor

20. Fixate chromosomes in solution of acetic acid and ethanol in 3:1 ratio

21. Store in ethanol at -20°C

4.1.4 PCR of genomic DNA for x43.1 probe

1. Prepare master mix for PCR:

   a. Water .... 936.1 µl

   b. Buffer 10x .... 115 µl

   c. dNTP 10 mM .... 23 µl

   d. primer F 10 mM .... 23 µl

   e. primer R 10 mM .... 23 µl

   f. Taq polymerase .... 11.5 µl

   g. Genomic DNA .... 18.4 µl

   h. TOTAL .... 1150 µl

2. Set up cycler

       a. Denaturation .... 4 min ....95°C

       b. Denaturation .... 20 s ....95°C

       c. Annealing .... 20 s ....56°C

       d. Elongation .... 1 min ....72°C

       e. Repeat step b-d 36 times

       f. Elongation .... 10 min ....72°C

3. Purify products of PCR with QIA quick gel extraction kit

4. Prepare agars gel with 1% agars concentration

       a. TAE buffer 1x ....60 μl

       b. Agar for electrophoresis ....0.6 g

5. Heat up the mixture in microwave oven to dissolve the agar

6. Cool down mixture to approximately 60°C and then add ethidium bromide

7. Pour mixture into form and let it form solid gel

8. Pipet premixed 5 μl of loading dye and 1 μl of sample into gel

9. Set electrophoresis electric source to 80 V and 30 minutes.
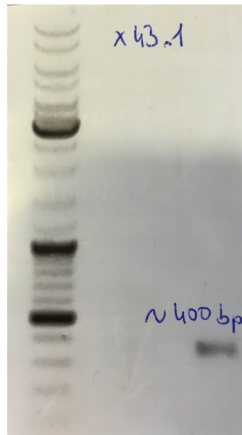
10. Check under UV light

Figure 11: X43.1 probe 350 bp long

11. Measure DNA concentration with UV-VIS spectrometer (e.g. Nanodrop from thermofisher)

4.1.5   Preparation of hybridization solution

Table 2: Ethylene carbanate hybridization mixture content

Ethylene carbonate mixture	
Chemical	V [μl]
Ethylene carbonate 15%	150
Dextran sulfate 20%	200
600 mM NaCl	100

10 mM Citrate buffer	100
ddH$_2$O	450
Total	1000

Preparation of 15% Ethylene carbonate from stock

15%........................150 μl

98%...........................x μl

$$x = \frac{15}{98} \cdot 150 = 23 \ \mu l \text{ of concentrated ethylene carbonate}$$

Equation

(9)

Preparation of 20% Dextran sulfate

20%........................200 μl

50%...........................x μl

$$x = \frac{20}{50} \cdot 200 = 80 \ \mu l \text{ of 50\% dextran sulfate}$$

(10)

Preparation of 600 mM NaCl solution

$Mr = 58.44 \, g/\text{mol}$

$c = 0.6 \, \text{mol}/\text{dm}^3$

$V = 0.01 \, \text{dm}^3$

$$c = \frac{n}{V} = \frac{m}{M \cdot V} \Rightarrow m = c \cdot M \cdot V = 58.44 \cdot 0.6 \cdot 0.01 = 0.35 \text{ g}$$

(11)

Table 3: Formamide free hybridization mixture content

Formamide free mixture	
Chemical	V [μl]
Dextran sulfate 50%	200
20x Sodium saline citrate	10
Salmon sperm DNA	50
ddH$_2$O	740
Total	1000

Table 4: Formamide hybridization mixture content

Formamide mixture	
Chemical	V [µl]
Deionized formamide 50%	500
Dextran sulfate 25%	200
20x Sodium saline citrate	100
Salmon sperm DNA	50
ddH$_2$O	150
Total	1000

Preparation of 15% formamide from 92% stock solution

50%.........................500 μl

92%...........................x μl

$$x = \frac{50}{92} \cdot 500 = 252 \ \mu l \text{ of 92\% formamide}$$

(12)

Preparation of 25% Dextran sulfate

25%.........................200 μl

50%...........................x μl

$$x = \frac{25}{50} \cdot 200 = 100 \ \mu l \text{ of 50\% dextran sulfate}$$

(13)

1. Prepare hybridization solution according to tables and equations

2. Store in well labeled tubes at -20°C

4.1.6   Labeling x43.1 DNA probe with fluorescence labeled dNTPs with NICK translation

1. Prepare NICK translation mixture according to table

   a. Note: Work on ice all the time

Table 5: NICK translation reaction mixture

NICK translation	V [µl]
DNA template (1-1.5 µg)	X
Water	14-X
10x NT labeling buffer	2
Labeled dNTPs mix	2
Enzyme mix	2
Total	20

2. Turn on cycler and set incubation at 15°C and

3. Incubate for 1.5 hours

4. Check the products with gel electrophoresis

5. Product has to be labeled with color of choice (eq. green for Alexa 488). Ethidium bromide has emits orange light under UV-light
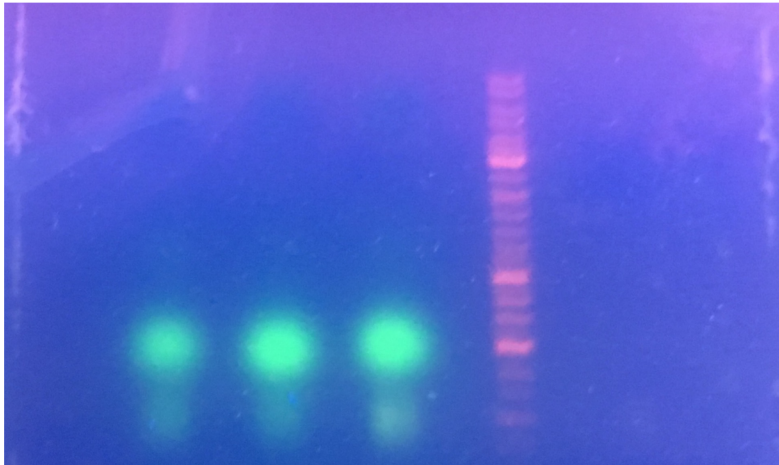

Figure 12: X43.1 probe labeled with Atto 488 under UV light

6. If the product isn't labeled properly, add 1 μl DNAse 1 and 1 μl 10x NT labeling buffer and repeat steps 3-5.
7. Precipitation of labeled product is recommended
   a. Add 0.1x sample volume of 3M sodium acetate
   b. Add 5x sample volume of 98% ethanol stored at -20°C
   c. Precipitate at -20°C overnight
   d. Set centrifuge for 14 000 rpm, -4°C, 30 minutes
   e. Discard supernatant without breaching the DNA pellet
   f. Add 70% ethanol
   g. Repeat the step d.
   h. Repeat the step e.
   i. Let the sample dry out bottom up for 5-10 minutes
   j. Dissolve the probe in ddH$_2$O
   k. Store at -20°C

As a probe for measuring the intensity of fluorescence light in FISH experiment Atto 488 was chosen. Atto 488 was chosen for high stability and shorter wavelength is better for resolution of signal.
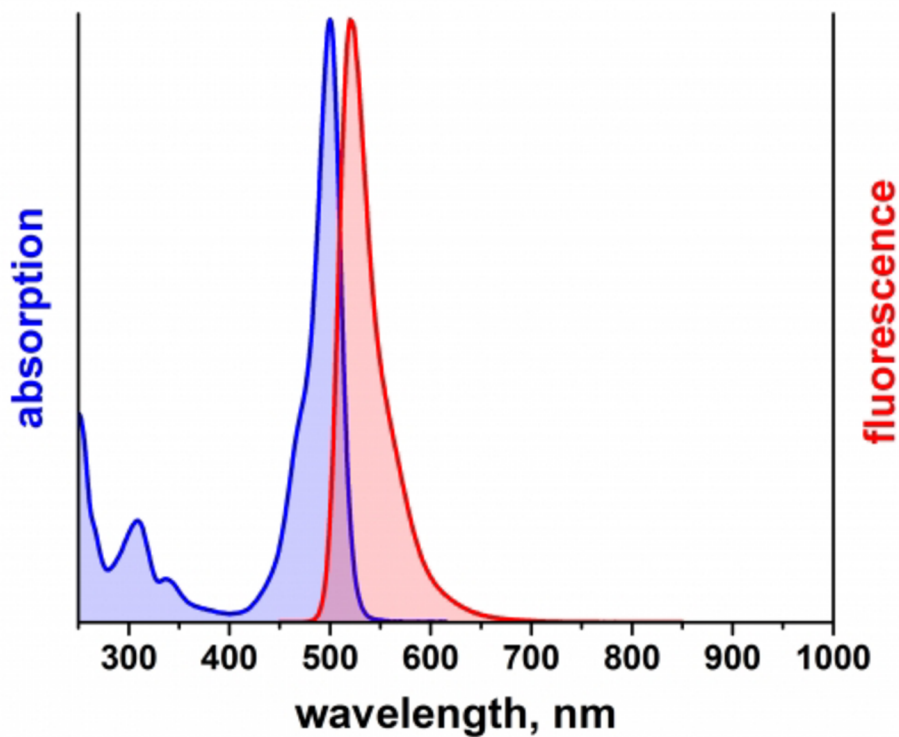
Figure 13: Excitation and emission spectrum of Atto 488

4.1.7 Fluorescence *in situ* hybridization

The process of hybridization is based on Schwarzer and Harrison

1. Let microscopic slides dry out after pulling them out of storage

2. Prepare solution of 2x SSC from stock solution of 20x SSC by diluting with watter and adjust pH to 7.2

3. Wash the microscopic slides 2 times for 5 minutes

4. Wash the microscopic slides in 3:1 ethanol-acetic acid solution for 10 minutes

   a. 3:1 solution should be prepared new for the process of hybridization

   b. Work in laboratory box with air vacuum

5. Pull out hybridization mixtures out of storage and incubate them on 37°C

6. Wash again 2 times for 5 minutes with 2x SSC

7. If the chromosomes are under cytoplasm, treat them with pepsin mixture

   a. Mix 5 µl of pepsin with 1 ml of 10 mM HCl

$$c_{\text{konc. HCl}} = 12\,\text{mol}/\text{dm}^3$$

$$c_{10\,\text{mM HCl}} = 0.01\,\text{mol}/\text{dm}^3$$

$$V_{\text{H}_2\text{O}} = 0.05\,\text{dm}^3$$

$$\underline{V_{\text{konc. HCl}} = ?\ \text{dm}^3}$$

$$c_{\text{konc. HCl}} \cdot V_{\text{konc. HCl}} + c_{\text{H}_2\text{O}} \cdot V_{\text{H}_2\text{O}} = c_{10\,\text{mM HCl}} \cdot \left(V_{\text{konc. HCl}} + V_{\text{H}_2\text{O}} \right)$$

$$c_{\text{konc. HCl}} \cdot V_{\text{konc. HCl}} + c_{\text{H}_2\text{O}} \cdot V_{\text{H}_2\text{O}} = c_{10\,\text{mM HCl}} \cdot V_{\text{konc. HCl}} + c_{10\,\text{mM HCl}} \cdot V_{\text{H}_2\text{O}} \tag{14}$$

$$c_{\text{konc. HCl}} \cdot V_{\text{konc. HCl}} - c_{10\,\text{mM HCl}} \cdot V_{\text{konc. HCl}} = c_{10\,\text{mM HCl}} \cdot V_{\text{H}_2\text{O}} - c_{\text{H}_2\text{O}} \cdot V_{\text{H}_2\text{O}}$$

$$V_{\text{konc. HCl}} \cdot \left(c_{\text{konc. HCl}} - c_{10\,\text{mM HCl}} \right) = c_{10\,\text{mM HCl}} \cdot V_{\text{H}_2\text{O}} - c_{\text{H}_2\text{O}} \cdot V_{\text{H}_2\text{O}}$$

$$V_{\text{konc. HCl}} = \frac{c_{10\,\text{mM HCl}} \cdot V_{\text{H}_2\text{O}} - c_{\text{H}_2\text{O}} \cdot V_{\text{H}_2\text{O}}}{c_{\text{konc. HCl}} - c_{10\,\text{mM HCl}}}$$

$$V_{\text{konc. HCl}} = \frac{0.01 \cdot 0.05 - 0 \cdot 0.05}{12 - 0.01} = 0.000\ 041\ 701\ l = \underline{41.701\ \mu l}$$

    b. Remove excessive 2x SSC from microscope slides, but don't dry them

    c. Put droplets of 10 mM HCl on slides and afterwards cover them with folia

    d. Put slides into wet-chamber and incubate at 37°C for time necessary to remove the cytoplasm (time of incubation depends on amount of cytoplasm)

    e. Wash slides 2 times for 5 minutes in 2x SSC

8. Move microscopic slides into solution of formamide and 2x SSC for 10 minutes

    a. Mix 10 ml of formamide with 84 ml of 2xSSC

    b. Work in fume hood

9. Prepare hybridization probe (mention volumes are per microscopic glass)

    a. 18 μl of hybridization mixture

    b. 1 μl of x43.1 probe

    c. Incubate in PCR cycler for 10 minutes on temperature necessary for DNA denaturation

    d. After incubation put straight on ice to preserve the probe in single strand state and let 5 minutes rest

10. Set hot plate for same temperature as cycler

11. Wash microscopic slides 2 times in SSC for 5 minutes

12. Prepare ethanol row (50%, 70% and 100% of the ethanol)

13. Dry microscopic slides using ethanol row (start form 50% and move slides after 2 minutes in each solution up to 100% of ethanol)

14. Let microscopic slides dry on air at room temperature

15. Put probe on the microscopic slide and cover gently with cover glass

16. Move slides on hot plate for 1-3 minutes

17. Move slides into the wet chamber

18. Hybridize the probe to target sequence at 37°C for 2, 4 or 16 hours

4.1.8 Probe washing

This step is designed to get rid of the un-hybridized probe or probe hybridized with low-strigency and takes place right after last step of hybridization process. Probe washing is necessary for strong signal of correctly hybridized probe and to lower background noise as much as possible.

1. Set the water bath thermostat at 57°C with 2x SSC in Coplin jar

2. Wash the microscopic slides 2 times in 2x SSC for 5 minutes at room temperature

3. Move slides into the Coplin jar in water bath for 20 minut

4. Move slides into the ethanol row

5. Dry the slides on air at the room temperature

6. Put 10-14 µl of DAPI + Vectashield on every slide, put cover glass on and apply force to remove excessive DAPI + Vectashield

7. Store at fridge at 4°C

4.1.9 Confocal microscopy

1. Move the slides out of the storage and temper them at 37°C

2. Turn on confocal microscope and computer

3. Set parameters of experiment in LASX software for Leica confocal microscopes

    a. Set power of laser power on 70%

    b. Set intensity of laser to 5%

    c. Set smart-gain on hybrid detector to 1250 V

    d. Set resolution for 16 bit

4. Acquire at least 10 images from every microscopic slide

5. After finished session clear the objective from immerse oil and turn off microscope

4.1.10 Image processing

1. Start the FIJI (ImageJ) application

2. Open project.lif file

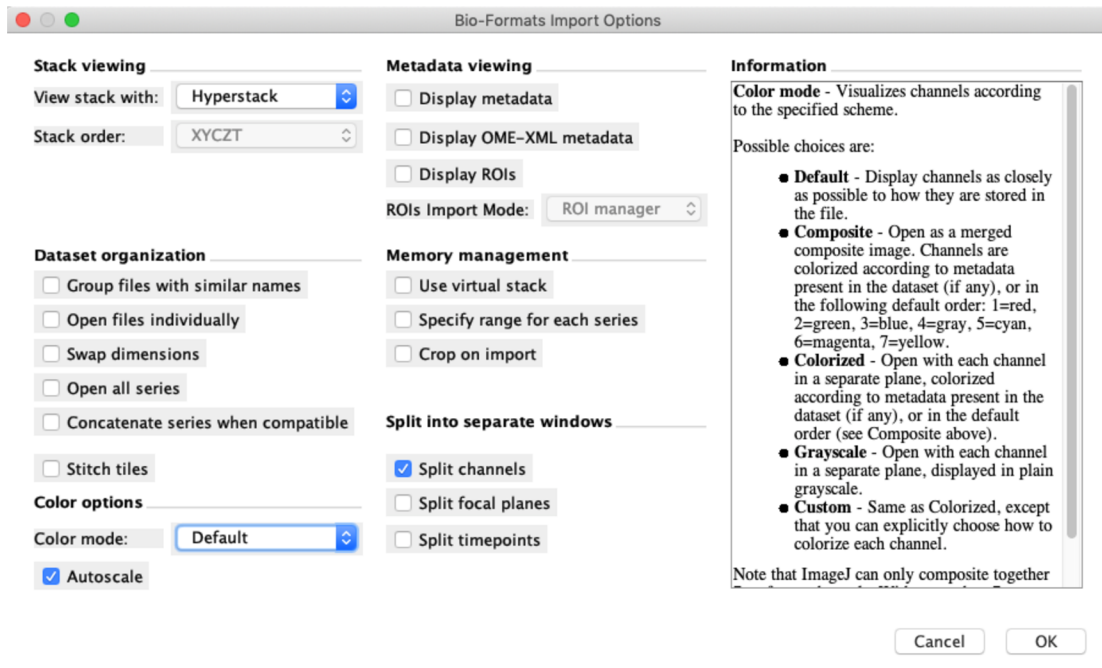3. Set the Bio-Formats Import Options accordingly:

Figure 14: Bio-Formats Import Option settings

4. Choose figures which you want to measure

5. Subtract background with rolling ball radius set on 10 pixels
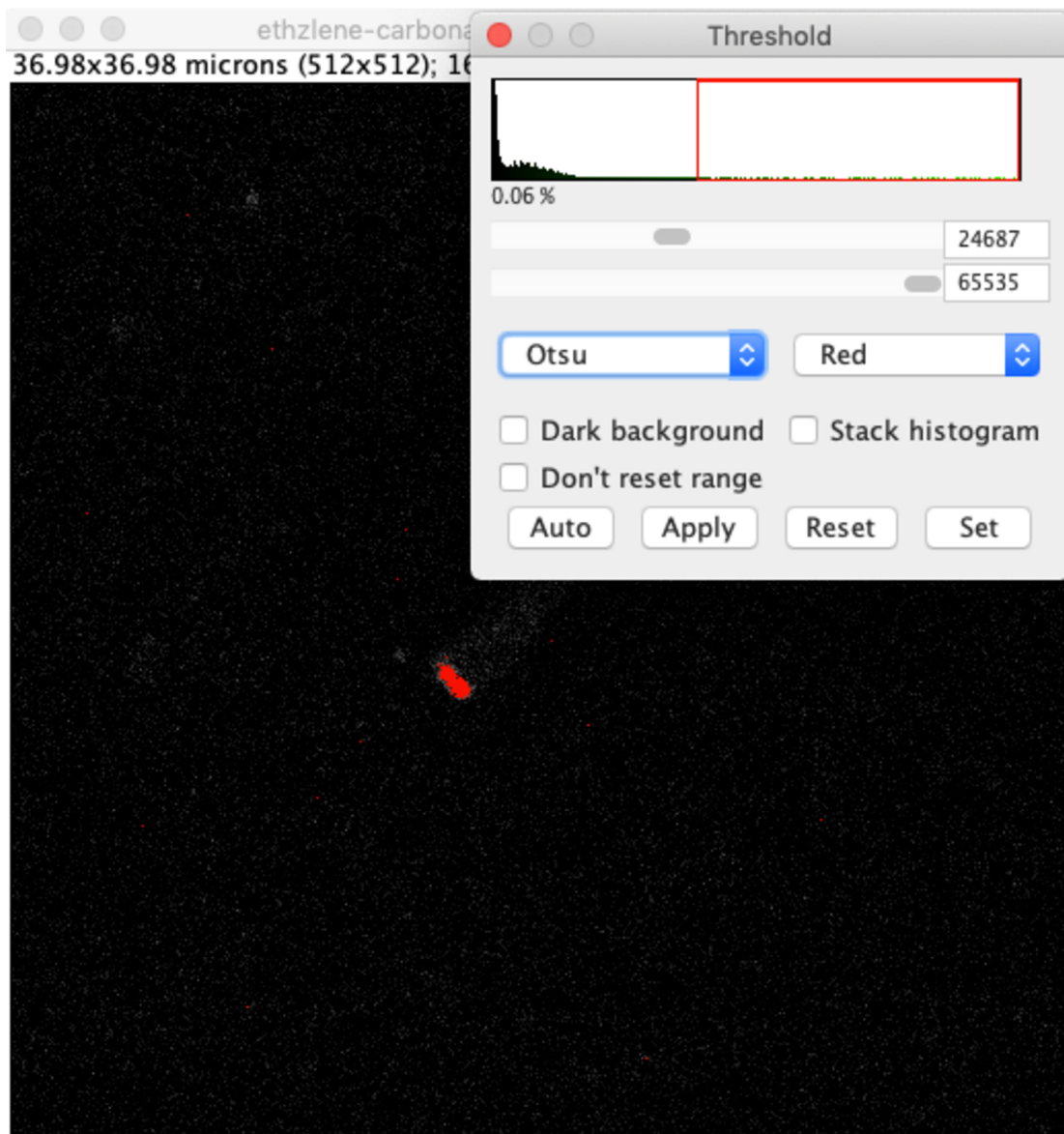
6. Adjust threshold

Figure 15: Threshold settings

7. Create selection

8. Open ROI manager

9. Add selection and split that selection so individual signals are accessible

10. Find signal of fluorescent probe on Y chromosome (If needed, mark two signals and press OR (Combine) to measure more than one signal unit)
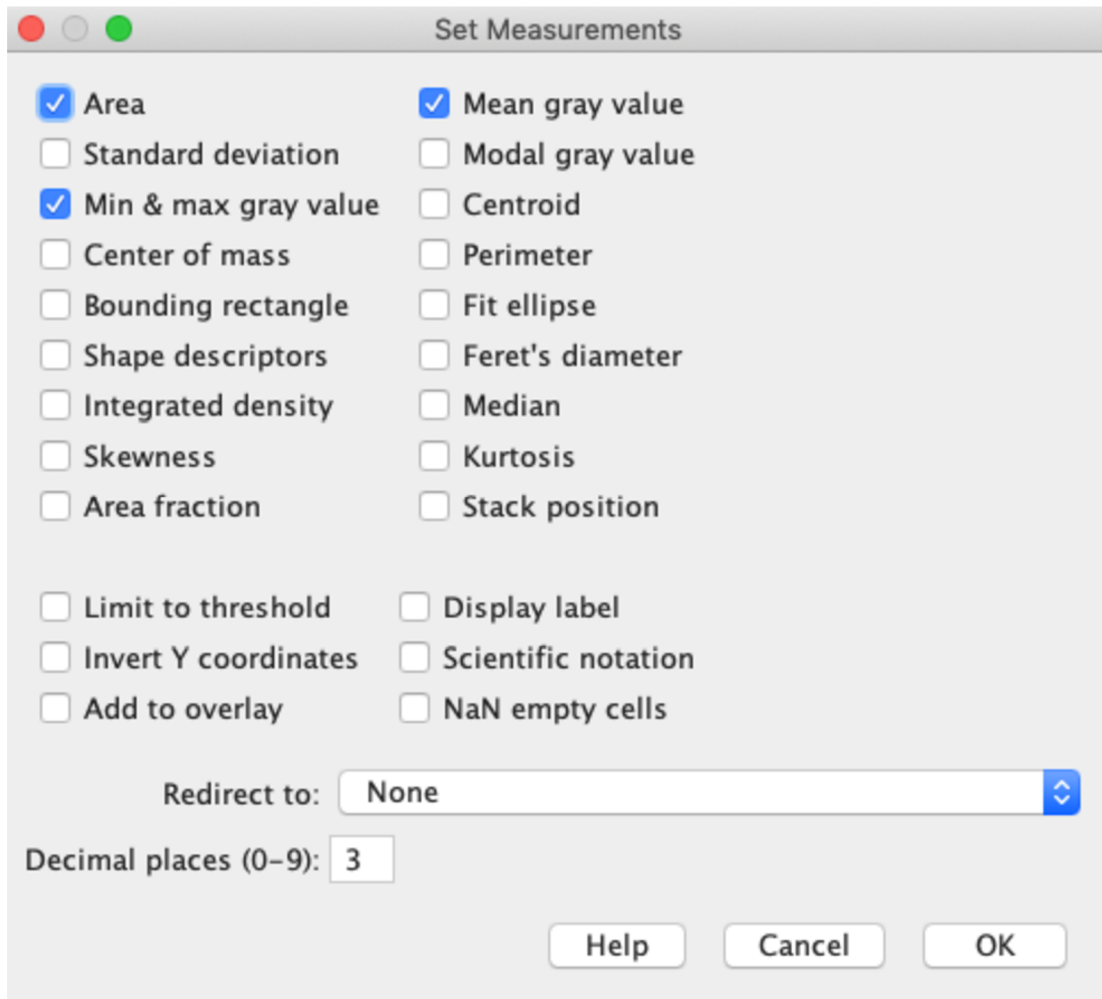
11. Reset threshold

12. Set measurement for:

Figure 16:Measurement settings

13. Measure the signal

14. Save results in .csv format

5 RESULTS

As the first step of this thesis was necessary to test melting temperatures for each hybridization mixture (formamide, EC and formamide free).. Concentration of DNA was chosen as 1 mg per ml [26]. Melting temperatures were obtained by differential scanning calorimetry. Sequence chosen for denaturation temperature measurement was 125 base pairs long. This length correspond to the typical lenght of nick translation products. The sequence contain 42.4% GC . Data obtained from DSC were used for temperatures calculation for denaturation during *in situ* hybridization.

Each mixture was measured in triplicate. From obtained data it is clearly visible that the temperature of denaturation differs between used solvents. Each solvent started to denaturate between 50-60°C and culminate over 70-85°C.

Shape of the melting curves is in agreement depending on the solvent and content of the hybridization mixture. The highest temperature (82.88 °C), achieved for formamide free mixture, is influenced by the low content of saline sodium chloride (SSC) ions and also by the fact that this mixture does not contain organic solvents (ethylene carbonate, formamide).

The impact of organic solvents is on other hand visible for formide and ethanol carbonate. These two mixtures has lower melting temperatures (75.30 °C for ethylene carbonate, 71.89 °C formamide) and high concentration of free SSC ions compared to formamide free mixture.

Beside all three mixtures contain dextran sulphate, which is increasing local density of DNA probe and viscosity of a hybridization mixture [13].
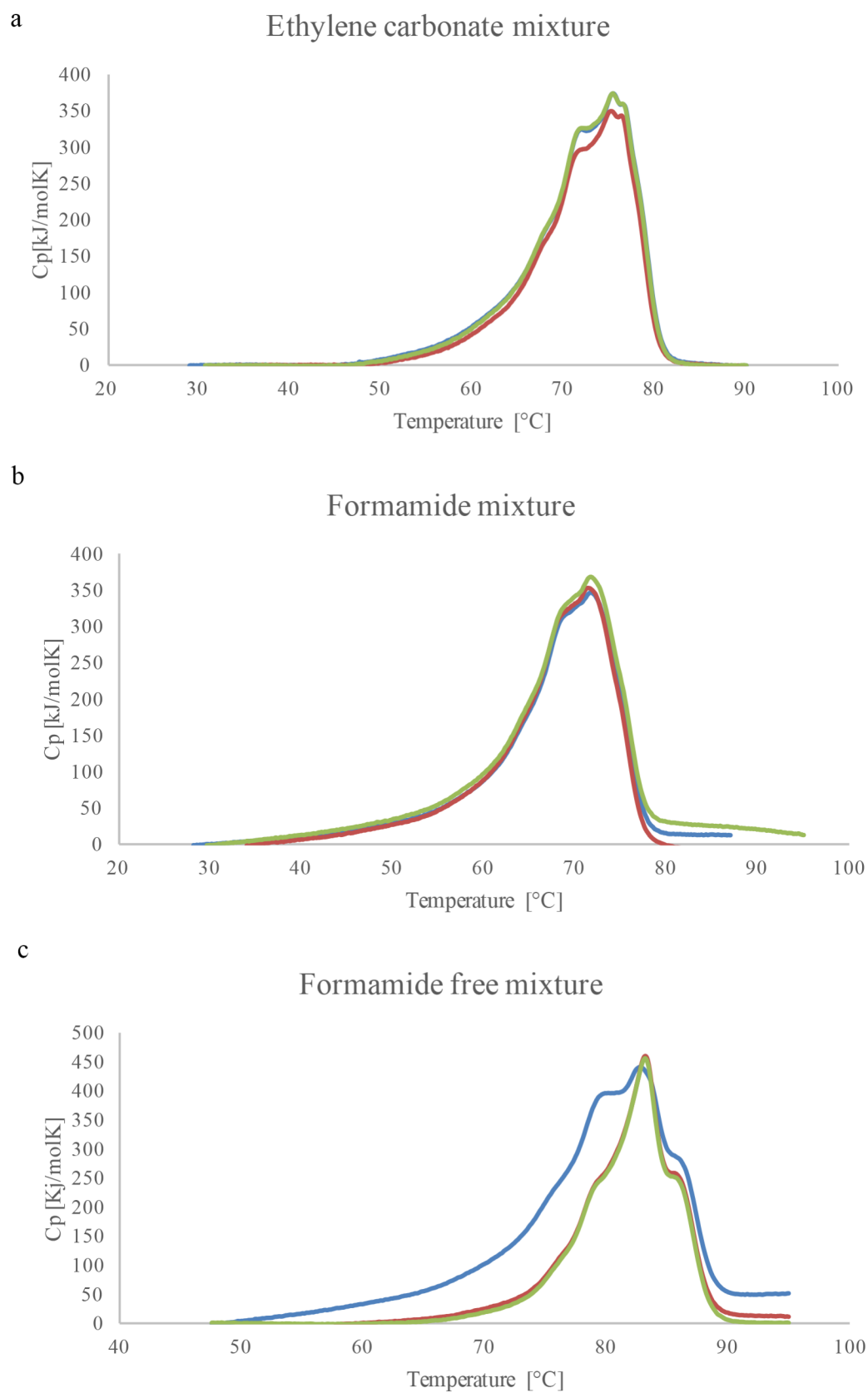
a

Ethylene carbonate mixture

b

Formamide mixture

c

Formamide free mixture

Figure 17: DSC melting curves for three different hybridization mixtures.

When comparing the Melting temperatures, the formamide mixture shows the lowest melting temperature out of these three hybridization mixtures at 71.89 °C. Second lowest melting temperature posses the ethylene carbonate mixture at average 75.30 °C. Highest temperature needed for DNA denaturation was measured in formamide free hybridization solution at 82.88 °C. From these temperatures we can assume that formamide solution at current composition is the most efficient when it comes to destabilization of secondary DNA structure. Formamide mixture shows that AT pairs denaturation comes about at 68.04 °C and CG pairs follows at 71.89 °C to finish the denaturation. Ethylene carbonate hybridization mixture start denaturation 71.27°C and finishes at 75.30 °C with additional peak at 76.27 °C. This additional peak is present in formamide free mixture but absent in formamide mixture. Formamide free mixture shows the highest denaturation temperature at 82.88 °C for CG pairs and 79.61 °C for AT.

Table 6: Thermodynamic parameters obtained by DSC

Table 7: Thermodynamic parameters

	EC	FM	FF
Tm [°C]	75.30	71.89	82.88
dH [kJ/mol]	4386.67	4126.67	4728
dCp [kJ/molK]	366.52	356.3189	453.162
dS [kJ/K]	185.76	349.49	191.36

Melting temperature, enthalpy and heat capacity were obtained trough software for data processing from DSC device. Entropy was calculated from equation (7).

Denaturation was endothermic process for all of hybridization mixtures. Highest amount of energy necessary to denaturate double stranded DNA was measured in formamide free mixture followed by ethylene carbonate and formamide mixture.

Entropy was the highest in the formamide mixture (349.49 kJ/K) by significant difference compared to formamide free (191.36 kJ/K) and ethylene carbonate mixtures (185.76 kJ/K). This result would suggest that the single strand DNA in the formamide mixture possess the smallest volume out of three hybridization mixtures. Higher local concentration for single stranded DNA are therefore increased moderately in ethylene carbonarte and formamide free mixture, and increased almost twice in formamide mixture (Table 7 - Entropy).
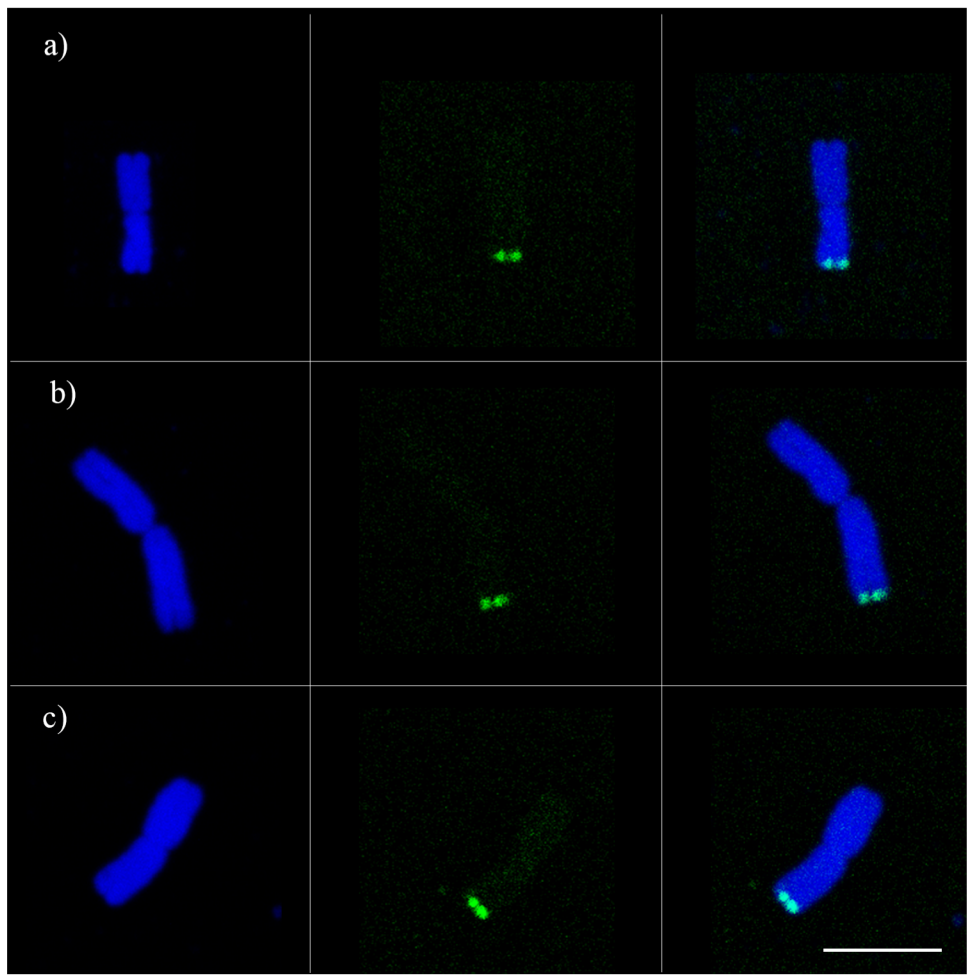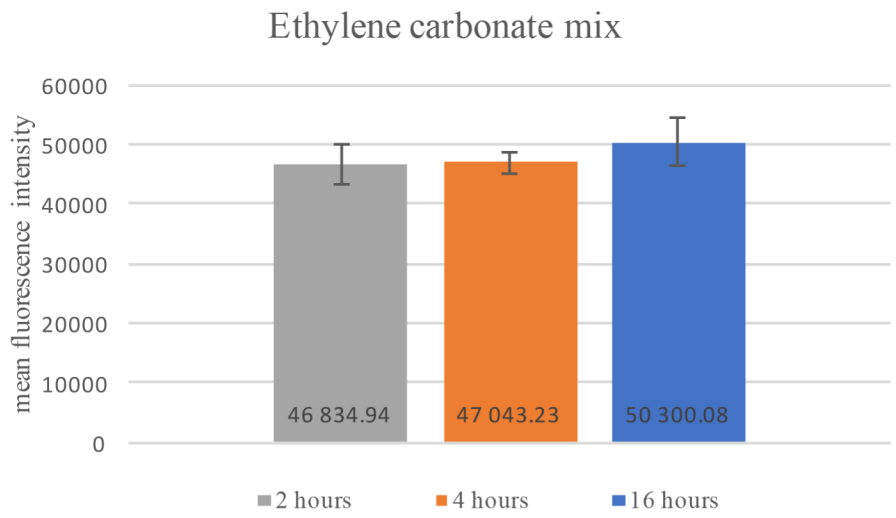
Figure 18: Comparison of mean fluorescence intensity of the ethylene carbonate mixture. a)2 hours hybridization interval, b) 4 hours hybridization interval, c) 16 hours hybridization interval. Scale bar = 5μm

Ethylene carbonate hybridization mixture provides strong signal even after 2 hours of hybridization. After 4 hours, the signal intensity is slightly higher and the highest yields form fluorescent probes were obtained after 16 hours hybridization. This hybridization solution provides best results for fast *in situ* hybridization with sufficient surface area. Signals from hybridization are well defined in all stages of hybridization time. In 2 and 4 hours hybridization times the results were influenced by even small amount of cytoplasm (Figure 10) that lead to more than 3x times decrease of signal intensity. The influence of cytoplasm decreased with longer hybridization time. Ethylene carbonate mixture provided less noise than other hybridization buffers without containing salmon sperm as blocking agent to unspecific signal.
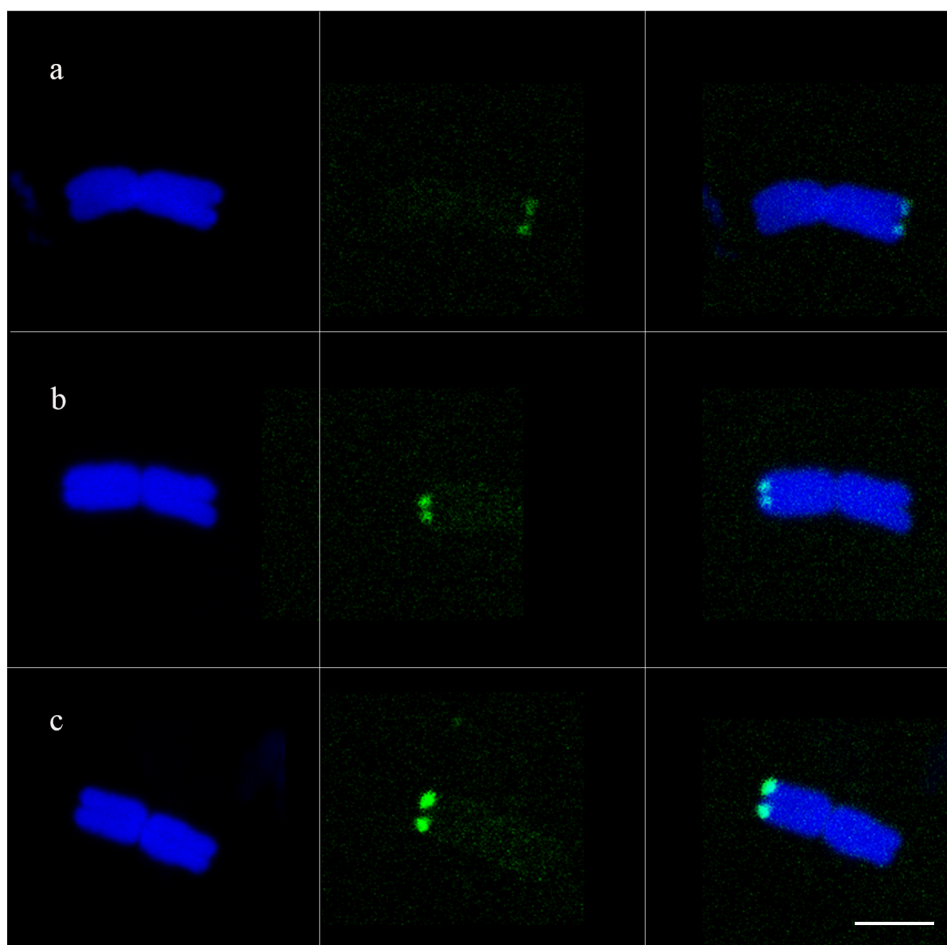
Formamide mix

Mean fluorescence intensity

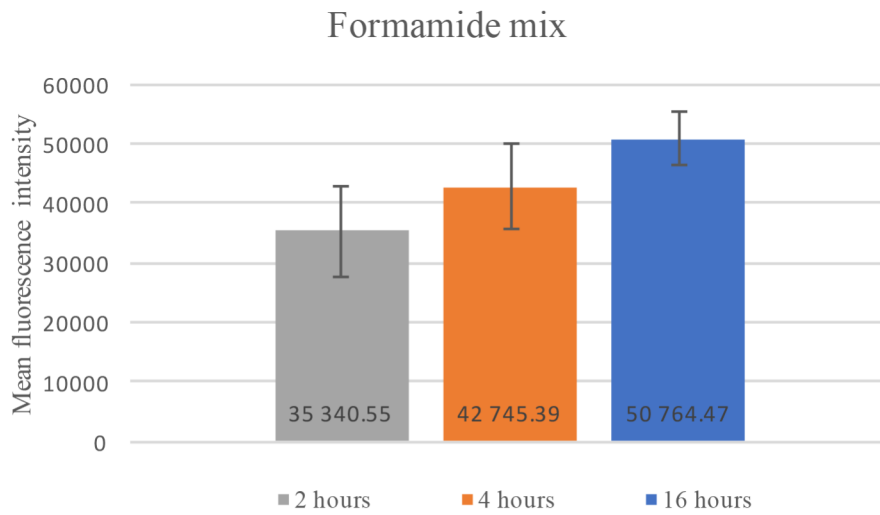- 2 hours: 35 340.55
- 4 hours: 42 745.39
- 16 hours: 50 764.47

Figure 19: Comparison of mean fluorescence intensity of the formamide mixture. a)2 hours hybridization interval, b) 4 hours hybridization interval, c) 16 hours hibridization interval. Scale bar = 5μm

Mean fluorescent intensity of formamide hybridization mixture is increasing significantly with hybridization time. Probe is well defined from 4 hours of hybridization time but signal lacks the in intensity when compared to 4 hours and especially to 16 hours hybridization interval. Background noise is more visible in formamide solution, especially at 4 hours hybridization time despite of using sheered salmon sperm to cover the repetitive sequences on genome.
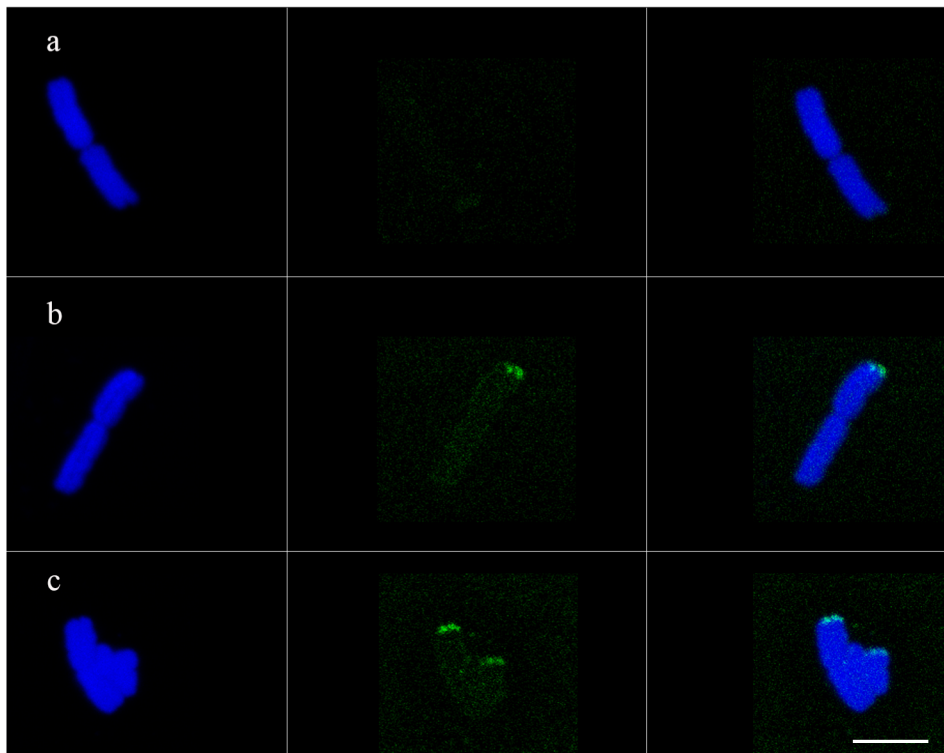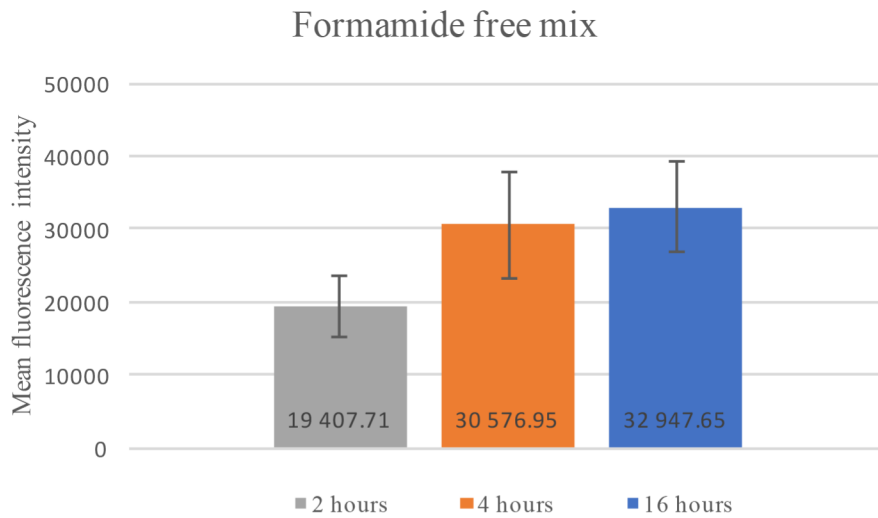

Figure 20: Comparison of mean fluorescence intensity of the formamide free mixture. a)2 hours hybridization interval, b) 4 hours hybridization interval, c) 16 hours hybridization interval. Scale bar = 5μm

The formamide free hybridization mixture was not able to produce well-defined signal at 2 hours of hybridization time and was hard to distinguish from background noise. The intensity was low and even in epi-fluorescent set up using mercury lamp with higher excitation power than laser beam was hard to visualize. The 2 hours hybridization became the limiting factor of the whole experiment. To even obtain signal in shortest time the intensity of laser had to be kept at 5% otherwise would be set lower for better resolution for other mixtures at 16 hours of the hybridization. The 4 hours hybridization is capable of providing signal that is defined. The 16 hours hybridization provides only slight improvement in signal intensity. The formamide free hybridization mixture was hilgly sensitive to cytoplasm in all measured hybridization times and only clean chromosomes were able to provide signal without significant amount of background noise.
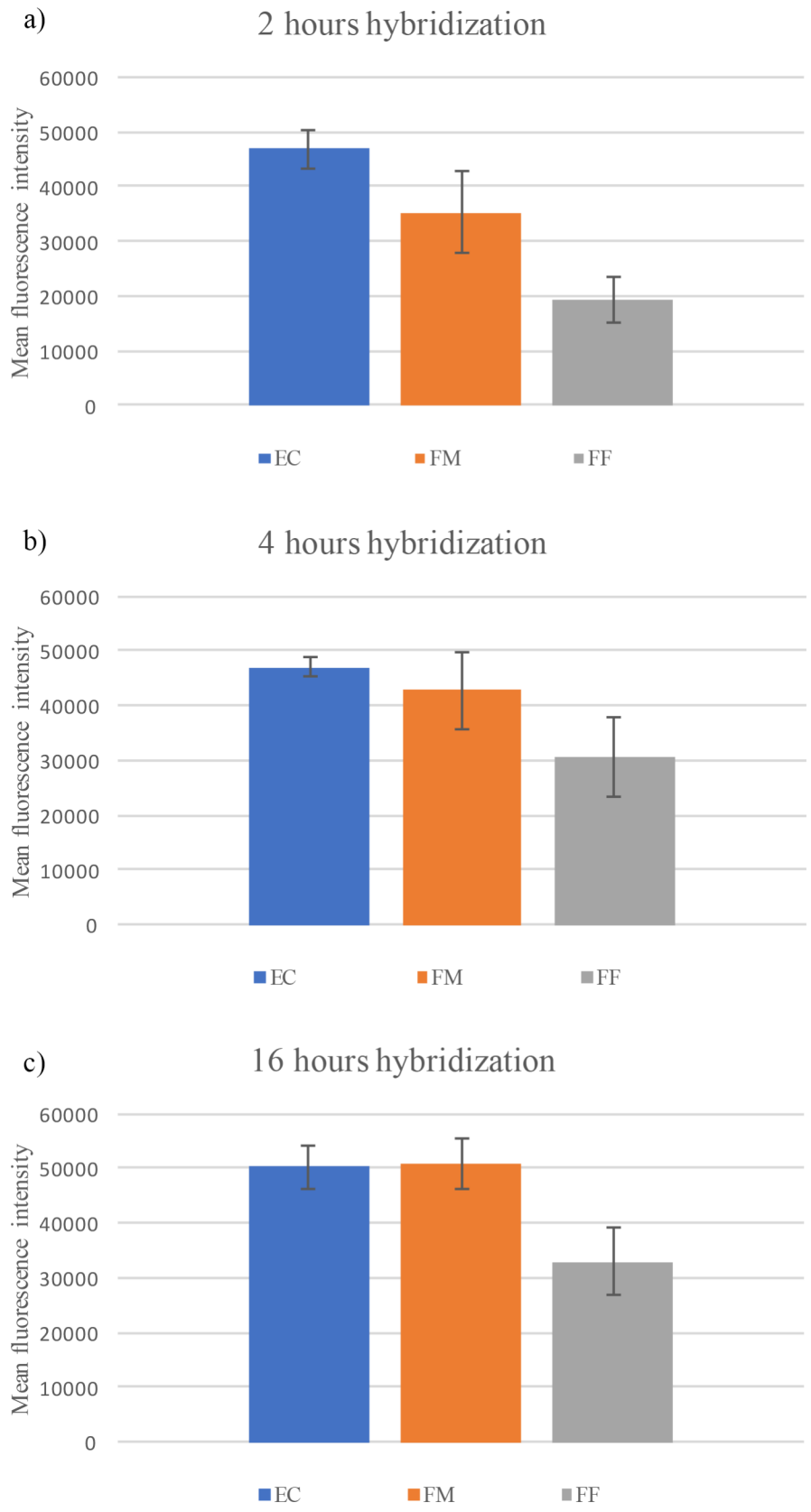
a) 2 hours hybridization

b) 4 hours hybridization

c) 16 hours hybridization

Figure 21: Comparison of hybridization mixtures across hybridization intervals

Comparing 2 hours hybridization, the fluorescence intensity the ethylene carbonate mixture provides best results, followed by formamide mixture and the formamide free mixture with lowest mean fluorescence intensity yields. The ethylene carbonate is the most consistents followed by the formamide free mixture. The formamide mixture is the most inconsistent out of these three hybridization mixtures.

The 4 hours hybridization mean fluorescent intensities are highest in ethylene carbonate. The formamide mixture has the second highest intensities. The difference between first and second is much smaller this at 4 hours hybridization. The formamide free mixture placed last again, but the difference between mean fluorescence intensity of formamide free and ethylene carbonate or formamide mixture is smaller at 4 hours then at 2 hours. Yield consistency is the highest in ethylene carbonate, the formamide stayed roughly the same but with increased signal intensity, the inconsistency in formamide free mixture rose as well producing the most inconsistent signal out of three mixtures at 4 hours hybridization time.

The 16 hours hybridization time is lead by formamide hybridization solution, which caught up with ethylene carbonate and produced slightly better results. Formamide free ybridization buffer provided only slight improvement over 4 hours hybridization time. Ethylene carbonate provided the most stabile result. Formamide and Formamide free improved significantly in consistency compared to 4 hours hybridization time.

6 DISCUSSION

Comparison of different hybridization solutions represents challenging task. The objective of this experiment was to provide unbiased conditions for all three mixtures. First aim was to determine the temperature of probe denaturation. Obtained values gave us idea on efficiency of non-covalent interaction that is responsible for DNA denaturation. The comparison of effects such as hydrogen bonds and base stacking were discussed in past, but not in relation to complex mixtures, such as hybridization buffers.

Faster hybridization times using the ethylene carbonate mixture, as proposed by Matthiasen were successfully proven by FISH experiments in this thesis [1]. The denaturation temperature was expected to be lower than in formamide mixture, but this assumption was not fulfilled, as the value of melting temperature was approximately 3 degrees higher than in formamide mixture. Nevertheless, the benefits of fast and non-toxic hybridization buffer are interesting and could be explored more in the future, especially regarding the low-copy or single copy gene sequences.

Formamide free hybridization mixture provided the lowest efficiency both in DNA denaturation temperature and fluorescence. Big advantage of this solution is its content, as most of used chemicals for this mixture are easy to handle and accessible. The further optimization of this solution is necessary, but hybridization of repetitive sequences with this hybridization buffer is plausible. My findings of this thesis corresponds with general demands of scientific community to further optimize conditions for formamide free hybridization mixtures [2].

Formamide hybridization mixture is well defined for *in situ* hybridization experiments. Despite the slower hybridization rate, results after 16 hours are comparable to ethylene carbonate mixture and shows even better potential for longer hybridization intervals. The strength of formamide mixture is the existence of many protocols for different applications across animal and plant specimens. The major drawback is the high toxicity, which leads to raised demands on safety of personnel dealing with this chemical and its solution and mixtures. The formamide hybridization mixture is the golden standard for FISH experiments [13], but I suggest as a stable replacement for shorter hybridization procedures. This could be valuable especially in clinical cytogenetics [1]

For further study of this topic I would like to investigate more the thermodynamic aspects of DNA in these solution and further optimize the formamide free and ethylene carbonate solution for better results of fluorescence yields and move from repetitive sequences to low-copy or single-copy genes, which are responsible for management of proteins or control pathways.

7 CONCLUSION

To sum up the results, the ethylene carbonate hybridization mixture is the fastest hybridization solution out of the three used. The melting temperature of DNA this hybridization solution was 75.27°C. The signal is well defined, consistent and strong from 2 to 16 hours of hybridization process. The background noise is not a big factor even from the shortest hybridization time. Only drawback is sensitivity to cytoplasm residues, which is pronounced especially in first 4 hours of hybridization.

Formamide solution is slower at hybridization rates but shows better potential for longer hybridization times as it overtook ethylene carbonate at 16 hours interval. Signal is well defined after 4 hours, but background noise is more evident even in case sheared salmon sperm was used as a blocking agent. Inconsistency of fluorescence intensity is more usual for this hybridization buffer than for ethylene caronate. Melting temperature of DNA in this buffer was the lowest (72.05°C).

Formamide free mixture was the least efficient out of the three tested and the DNA melting temperature was the highest (82.88°C). The 2 hours hybridization is possible but the signal is hard to distinguish form background noise. At the 4 hours hybridization, formamide free mixture could be regarded as more competitive, but longer hybridization time resulted in only slight improvement.

Enthalpy values did not provided information about interaction of DNA and hybridization solution, because of the differences between energies necessary to denature DNA helix. To obtain this information, it would be necessary to either equalize the melting temperature or create calibration curve. Isothermal titration calorimetry experiment might be helpful, to solve this issue.

Entropy of system was higher in every hybridization mixture, which was partially caused by increased temperature. The differences between individual hybridization mixtures suggest that the strongest aggregation of ssDNA occurs in the formamide mixture. Folding of DNA is probably less prevalent in ethylene carbonate and formamide free mixture.

The thesis shows benefits of individual mixtures and when is favourable to use ethylene carbonate or formamide as hybridization mixture. Obtained data serve us as a good starting point for further optimization of hybridization conditions and contents of selected buffers. Thermodynamic data needs to be investigated with different methods to gain better insight into DNA alterations and denaturation process that take place hybridization mixtures.

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8 LIST OF ABREVIATONS USED

FISH – Fluorescence *in situ* hybridization
DNA – deoxyribonucleic acid
dsDNA – double strand DNA
ssDNA – single strand
bp – base pair(s)
A - adenine
T - thymine
C - cytosine
G - guanine
DSC- differential scanning calorimetry
U –internal energy
Cp – heat capacity
H –enthalpy
S - entropy
P - preassure
V - volume
UV-VIS – ultra violet – visible
dNTP –deoxynucleoside triphosphate
SSC – sodium saline chlorid
EC – ethylene carbonate
FM – formamide
FF – formamide free
LSCM – laser scanning confocal microscopy
ROI – region of interest
PCR – polymerase chain reaction