UPPSALA
UNIVERSITET

UPTEC X 16 021
Examensarbete 30 hp
Juni 2016

Promoter regulation

designing cells for biotechnological applications

Mikael Andersson Schönn

UPPSALA UNIVERSITET

Degree Project in Molecular Biotechnology

Masters Programme in Molecular Biotechnology Engineering,
Uppsala University School of Engineering

UPTEC X 16 021	**Date of issue 2016-06**

Author

Mikael Andersson Schönn

Title (English)

Promoter regulation – designing cells for biotechnological applications

Title (Swedish)

Abstract

The filamentous cyanobacteria *Nostoc punctiforme* ATCC 29133 is a model species for development of sustainable production methods of numerous compounds. One of its unique features is the anaerobic environment of the strains nitrogen fixing heterocyst cells. To be able to properly utilize this environment, more knowledge regarding what regulates cell specific expression is required. In this study, three motifs of the NsiR I promoter of *Anabaena sp.* PCC 7120 was studied in this system utilizing YFP-fluorescence as a reporter to determine their impact on spatial expression pattern. Investigations were performed on immobilized cells with the use of confocal microscopy and results point towards sigma factor regulation.

Keywords

Cyanobacteria, heterocyst, promoter, specificity, confocal microscopy, immobilization

Supervisors

PhD Karin Stensjö
Uppsala University

Scientific reviewer

PhD Pia Lindberg
Uppsala University

Project name	Sponsors
Language **English**	Security
ISSN 1401-2138	Classification
Supplementary bibliographical information	Pages **66**

Biology Education Centre Biomedical Center Husargatan 3, Uppsala
Box 592, S-751 24 Uppsala Tel +46 (0)18 4710000 Fax +46 (0)18 471 4687

Promoter regulation – designing cells for biotechnological applications

Mikael Andersson Schönn

Populärvetenskaplig sammanfattning

I och med det ständiga hotet global uppvärmning och miljöförstöring utgör i dagens samhälle, höjs ständigt nya röster för att utveckla en grönare förnybar industri. I fronten av denna utvecklingsvåg finner vi cyanobakterier. Dessa organismer är speciellt intressanta då de är kapabla till att växa utan näst intill några tillsatta näringsämnen. En av de mest intressanta arterna är den filamentösa cyanobakterien *Nostoc punctiforme* vilken består av två olika celltyper, vegetativa celler och heterocyster. Heterocysterna är specificerade celler vars huvudsakliga funktion är att fixera kväve. Denna egenskap kräver att dessa celler har ett fullständigt syrefritt innanmäte vilket är intressant vid produktion av visa specifika ämnen. För att kunna utnyttja denna miljö måste vi förstå vad som styr cellspecificiteten i genuttrycket. Detta görs generellt av en så kallad promotor. I den här rapporten undersöks specifikt hur tre olika promotormotiv påverkar heterocystspecificitet.

Undersökningen utfördes genom att kombinera de olika motiven i ett syntetiskt promotorskelett på en plasmid som kodar för ett gult fluorescerande protein (YFP). Därefter analyserades uttrycket i levande celler med hjälp av konfokalmikroskopi och kvantitativa fluorescensmätningar.

Resultaten inkluderar skapandet av en fullständigt syntetisk promotor med heterocystspecifikt uttryck som teoretiskt sett ska kunna utnyttjas utan att på något sätt interferera med nativa promotorer. Vidare upptäcktes att det tre individuella motiven på något sätt samverkar vilket antyder att alla heterocystspecifika promotorer regleras på samma sätt. Slutligen observerades även indikationer på att ursprungspromotorn ursprungligen påverkas av tillgången till lösliga kvävemolekyler i omgivningen för att sedan skifta till någon annan typ av reglering.

Examensarbete 30 hp
Civilingenjörsprogrammet i Molekylär bioteknik
Uppsala universitet, juni 2016

Extended abstract

With the ever looming threat of climate change, calls for a more environmentally sustainable industry are being heard all over the globe and production of precious compounds through chemical synthesis is being challenged by environmentally friendly microbial factories. This green revolution does however call for further development and understanding of the potential production hosts available today to fully be able to utilize their potential.

In the forefront of development, we find cyanobacterial systems. Their photoautotrophic capabilities enable production of a wide range of products at a low upkeep cost. Among the most efficient of these systems, we find the filamentous strain *Nostoc punctiforme* ATCC 29133. The filaments of *N. punctiforme* feature a two component system with energy producing vegetative cells and nitrogen fixing heterocysts. Out of these, heterocysts are especially interesting due to their anaerobic internal environment, enabling them to host and produce oxygen sensitive machinery and compounds.

However, to properly be able to utilize this environment there is a need to discover a way to efficiently move gene expression into the heterocyst without disrupting native machinery. This makes investigation into what regulates heterocyst-specific promoters such as the NsiR I promoter of *Anabaena sp*. PCC 7120 very interesting. In this report we have investigated the function of three sequence motifs found to be conserved in this promoter, and a set of 220 other heterocyst-specific promoters, with the purpose of unlocking what creates this kind of specificity.

The investigation was performed using a set of genetically engineered strains using different combinations of the conserved promoter elements to drive the expression of fluorescent YFP. The expression pattern of the strains was then analyzed with the use of confocal fluorescence microscopy and quantitative fluorescence measurements.

The results presented in this report include the creation of a fully functional entirely synthetic promoter with the use of consensus sequences and a scrambled NsiR I shell. This promoter would theoretically function without affecting native systems since it completely lacks native elements. Additionally we discovered that all three conserved regions have a noticeable effect on promoter expression, indicating that they regulate expression together. The pattern displayed sequence-wise leads to the belief that the promoter might be regulated by a sigma factor which subsequently would be the case for all heterocyst-specific promoters. Finally, it is discovered that the NsiR I promoter shows evidence of split regulation. Switching out one of the conserved regions changes the expression pattern in a manner that indicates that the promoter is originally regulated through the presence of nitrogen, but is later taken over by heterocyst-specific regulation.

The evidence presented in this report opens up the possibility for a wide range of interesting studies that in the future could help us to fully understand the mechanisms behind heterocyst-specificity.

Table of contents

Abbreviations

YFP	Yellow fluorescent protein
TSS	Transcription start site
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
UP	Upstream promoter
CTD	C-terminal domain
UAS	Upstream activating site
DSR	Downstream sequence region
DIF	Differentiation
GFP	Green fluorescent protein
RBS	Ribosome binding site
PCR	Polymerase chain reaction
DMSO	Dimethyl sulfoxide
PS	Photosystem
pro-heterocyst	Prospective heterocyst
pre-heterocyst	Predetermined heterocyst
chl a	Chlorophyll A

1. Introduction

1.1 Project outlines

The aim of this master's degree thesis has been to investigate and evaluate the importance of specific motifs of the *Anabaena sp*. 7120 NsiR I promoter in the filamentous cyanobacteria *Nostoc punctiforme* ATCC 29133 with respect to transcriptional regulation and heterocyst-specificity. In particular, this has involved the construction of a fully functional, synthetic, heterocyst-specific promoter as well as several functional mutant promoters, changed with regard to regions interesting for heterocyst-specificity. The effects on gene expression in these strains have been examined through yellow fluorescent protein-(YFP-) mediated fluorescence studies utilizing confocal microscopy and plate reader fluorescence measurements.

The work itself can be considered as a continuation of previous published work (Li *et al.*, 2015), where the NsiR I promoter was scrutinized with regard to the transcription start sites, eventually leading to the identification of regions believed to be important for heterocyst-specific expression. In combination with additional related studies on cell specificity in promoters (Mitschke *et al.*, 2011; Muro-Pastor, 2014) and a weblogo investigation (Crooks *et al.*, 2004)(Appendix A) of conserved regions in a number of cyanobacterial promoters, consensus sequences for the pribnow-box as well as the discovered -35 region differentiation motif was procured. With these sequences and the native NsiR I promoter as a base, a range of promoter constructs was designed with the purpose of investigating the effect on cell specificity caused by each region. Synthesized copies of the desired constructs were ordered commercially and cloned by traditional restriction-based means into the YFP-carrying pSCR_AW_YFP vector followed by transformation by electroporation into wild type *N. punctiforme*. Finally, the fluorescence patterns of the different strains were examined to determine cell specificity, promoter strength and temporal expression pattern.

This study is of importance in the development of genetic tools to optimize the usage of the cyanobacterial chassis as model system. With increased knowledge regarding the transcriptional regulation and expression pattern of heterocyst-specific promoters, we open up the possibility to engineer synthetic counterparts that function without interference with the native system. Such promoters can be valuable in the expression of exogenous protein systems that required the anaerobic environment of the heterocyst. An example of such a system would be the introduction of an exogenous hydrogenase to promote hydrogen evolution for biofuel creation purposes.

1.2 *Nostoc punctiforme* as a host organism

With the recent surge towards a more environmentally sustainable industry, synthetic approaches to compound production are being challenged by microbial factories. In the forefront of research in this revolution we find cyanobacterial systems. Due to their aquatic photoautotrophic behavior, they serve as an excellent candidate for large scale production since they fix carbon directly from the air, gain energy from sunlight and can grow freely in water, removing three of the largest costs in cell cultivation. Moreover many cyanobacteria can fix nitrogen, removing this supplement demand as well.

One of the most interesting cyanobacterial strains when it comes to industrial application is the filamentous *N. punctiforme.* In addition to previously mentioned advantages, it also features a fast growth rate, relative genetic simplicity compared to higher organisms as well as a well-developed genetic toolbox (Heidorn *et al.*, 2011). However, the most interesting feature when it comes to filamentous strains is the presence of heterocysts.

Heterocysts are specialised cells that appear at roughly every tenth position in a filament during nitrogen starvation. Differentiation into this type of cell occurs gradually and they basically serve to fix atmospheric nitrogen and support the whole structure with nitric compounds when these cannot be found otherwise in the environment (Meeks *et al.*, 2001). A fully developed heterocyst loses the ability to divide into new cells so that there is not an overdevelopment of this cell type. In difference from the more common type of cell known as vegetative cells, the heterocysts have partly degraded most of the components specific for photoautotrophic life forms, among them photosystem II, in exchange for their nitrogen fixing ability (Meeks *et al.*, 2001). As such, the heterocysts are unable to photosynthesize and do not fix carbon, which in turn means that they have to procure energy in form of reduced carbon from neighboring cells. Morphologically, the heterocysts are bigger and rounder in shape than the more oblong vegetative cells and have a much thicker triple layered cell wall (Tamagnini *et al.*, 2002; Thiel, 2004). This along with high respiration and expression of enzymatic antioxidants helps the heterocysts to create an anaerobic intracellular environment which is crucial for the function of the enzymes involved in nitrogen fixation and hydrogen metabolism.

This type of environment is particularly interesting when producing compounds sensitive to oxygen or utilizing oxygen sensitive machinery. This could for example be introduction of an exogenous hydrogenase for hydrogen production or manipulation of the native nitrogenase to shift production rates of molecular hydrogen.

1.3 Cyanobacterial promoter systems and transcriptional regulation

Promoters are short DNA regions of roughly ~100-1000 base pairs (bp) located just upstream of the transcription start site (TSS) of a coding gene. The main purpose of this structure is to help initiating transcription of DNA into RNA through provision of a binding surface for the necessary polymerase and cofactors (Browning and Busby, 2004). In bacterial systems, recruitment of the RNA transcribing polymerase is mediated by proteins known as sigma factors, along with transcriptional elements with either activating or repressing effect (Browning and Busby, 2004).

Due to the conserved behavior of these regions, all prokaryotic promoters share a general consensus sequence outline. Using the TSS as a point zero, we find two especially conserved regions at -10 and -35 bp upstream respectively (Browning and Busby, 2004). As shown in figure 1, these regions are of high importance for the specific binding of the active sigma factor domains to the target promoter, prior to association of the RNA-polymerase holocomplex. The -10 region or Pribnow-box is an analog to the eukaryotic TATA-box and features a similar adenine and thymine rich motif with a consensus sequence of TATAAT (Pribnow, 1975). In addition to serving as a recognition binding site for the RNA-polymerase, the AT-rich composure of this region simplifies splitting of the helix to allow access to the single DNA strand (Yakovchuk *et al.*, 2006). In difference, the -35 region with its TTGACA consensus region has no known extra features other than the specific binding of the polymerase.

Finally, the last particularly common genetic feature is found upstream of these core regions and is known as an upstream promoter element or UP-element (Browning and Busby, 2004). This feature is not as strictly conserved but most of the time heavily favors the A and T bases. Through binding the polymerase sigma-C-terminal domain (CTD) (figure 1), it helps to greatly enhance the levels of produced transcript (Gruber and Gross, 2003).

Although heavily conserved, these regions are seldom found in the complete consensus version in nature and experiments have indeed proved these to be less effective than versions with single point mutations (Scholten and Tommassen, 1994). Regardless of this, the recognition regions are still highly specific for individual sigma factor types and crosstalk is very rare (Browning and Busby, 2004).

One of the main expression-regulating methods in most prokaryotes is the expression of the correct sigma factors. Without the completely correct recognition regions present in the RNA-polymerase complex, it is impossible for the polymerase to properly bind to the promoter and subsequently transcribe the gene (Browning and Busby, 2004). Some sigma factors are expressed continuously, known as primary sigma factors, and as such feature the sequence recognition of the most fundamental pribnow-box and -35 region of the host organism, while others are solely expressed during particular stimulus (Gruber and Gross, 2003). Due to this peculiarity, one can often use the sequences of these regions to determine the expression requirements of the promoter. Additionally, this shows that sigma factor regulation typically relates to a more fundamental change in the cell, such as sporulation or other defensive mechanisms, and always affects several promoters' expression at once (Flanagan *et al.*, 2016). Naturally, sigma factors are subjected to regulation themselves as well and can be sequestered by anti-sigma factors under certain stimulus (Flanagan *et al.*, 2016).

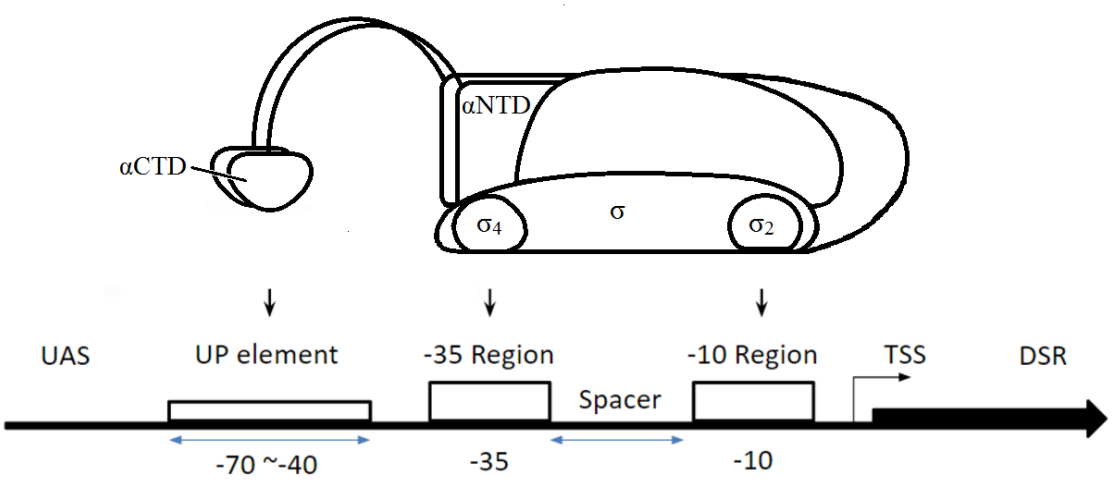


Figure 1: Description of the general outline of a bacterial promoter, featuring an RNA-polymerase with an associated sigma factor and the DNA with the conserved binding regions. Left to right; Upstream activating sequence (UAS), sigma-C-terminal domain (αCTD) binding to the DNA UP regulating element, fourth sigma-subunit (σ_4) binding to the -35 region, ~17 bp spacer, second sigma-subunit(σ_2) binding to the -10 pribnow-box, transcription start site (TSS) and downstream sequence region (DSR). Adapted from (Gruber and Gross, 2003).

Except for this general sigma factor-related regulation, there are several more direct ways to regulate individual promoters involving regions known as operators. Some promoters completely lack operators and are as such expressed evenly throughout the lifetime of the cell. Such promoters are known as constitutive promoters and typically regulates the expression of housekeeping genes of some sort which are absolutely crucial for cell survival (Huang and Lindblad, 2013). Another kind, known as inducible promoters, act under either induction or repression of the operator region. The regulation in this case is caused by presence of specific compounds or proteins. The regulatory compounds can range from primary metabolites to heavy metal ions depending on the gene regulated and it is not uncommon that an induced promoter also features a coupled repressor (Kennel *et al.*, 1977).

It is important to note that many promoters adhere to a number of these different regulation effects and as such it is very hard to predict the behavior of a promoter *in vitro* (Copertino *et al.*, 1997). This is also the main reason we need to study transcriptional regulation very carefully when investigating new potential promoters for synthetic introduction as well as for *in vivo* expression analysis.

1.4 The NsiR I promoter, conserved motifs and heterocyst-specificity

The NsiR I promoter is a very small promoter native to the filamentous cyanobacterial strain *Anabaena sp*. PCC 7120, which codes for an heterocyst-specific sRNA named nitrogen stress inducible RNA1 (Ionesco *et al.*, 2010). The promoter is natively found in twelve tandem repeats and expression occurs after roughly 4-6 hours of nitrogen starvation and predates any morphological changes in the cells during cell differentiation (Muro-Pastor, 2014). As such, it can be used as a very early indication of heterocyst formation. In addition to this, the promoter consist of a what is believed to be the minimal sequence requirements to induce heterocyst-specific expression I.e. features in the promoter causes no significant expression outside of immature and mature heterocysts (Muro-Pastor, 2014).

In a related comparative promoter study performed by (Li *et al.*, 2015) regarding the orthologous promoter pair of Npun_R5799 in *N. punctiforme* and alr3808 in *Anabaena sp*. PCC 7120, it was discovered that the known heterocyst-specific expression of alr3808 was mediated by a previously unknown distal TSS upstream of the proximal one. Closer investigation of the promoter associated with this TSS produced a similar premature expression behavior when coupled to green fluorescent protein (GFP) as the NsiR I promoter previously described. Subsequent sequence analyses of the two sequences lead to the discovery of the presence of a conserved differentiation- (DIF) motif (TCCGGA) between the two promoters. This motif had previously been associated with heterocyst differentiation in filamentous cyanobacteria (Mitschke *et al.*, 2011). Additionally, sequence alignment of several other promoter regions, orthologous to the alr3808, with the original one identified a highly conserved pribnow-box (TAGAGT) (Li *et al.*, 2015).

These discoveries motivated further analysis of the comprehensive *Anabaena sp*. PCC7120 promoter dataset produced with the use of differential RNA-sequencing by (Mitschke *et al.*, 2011). Multiple sequence alignment and a weblogo study (Appendix A) of the 220 promoters, that had been clearly related to heterocyst-specificity in this study, proved a general conservation of an extended pribnow-box ranging from -10 to the TSS (GAAAAATTTAA) and the -35 DIF motif, as well as fairly strong conservation of an AT-rich upstream region that potentially could be an UP-element (TGTTCATAAATAACCAGCATAATC).

The high conservation in these regions clearly indicate that regulation of these promoters are performed in a similar fashion, leading to the hypothesis that all heterocyst-specific promoters have at least one single major regulatory mechanism in common. The fact that the most conserved domains differ from the general prokaryotic consensus regions (Mitschke *et al.*, 2011) further raises the suspicion that this factor might actually be a separate sigma factor. As such, it is of interest to investigate the effect of each of these regions on heterocyst-specific expression separately to determine if they are all required for specificity. Finally, this discovery also opens up the possibility for the construction of a fully synthetic promoter that would not interfere with existing cellular machinery and as such be a valuable tool for genetic engineering in heterocysts.

1.5 Fluorescence reporters, yellow fluorescent protein and confocal microscopy

The fluorescent phenomenon is a type of luminescent emission and is caused by a shift in the energy levels of electrons in the valence orbital of the studied compound (Lakowicz, 2013). When compounds with certain characteristics, either containing several aromatic groups or rich in molecular pi-bonds, are exposed to electromagnetic radiation of specific wavelengths, energy is absorbed causing one or several electrons in the outer molecular orbital to become excited and move to a higher energetic state. This state is however not permanent and as soon as no more energy is provided, the electron relaxes to its original state and in the process releases light. The light released from the fluorophore is of lower energy and longer wavelength that that which was originally absorbed, an effect known as stokes shift (Lakowicz, 2013).

When performing expression experiments *in vivo,* one is faced with several problems caused by the environment. One of the foremost of these is to find a functional reporter system that fills the specific needs of the project without interfering too much with cell growth and functions. Fluorescent reporter proteins have been commercially available for a long time and come in a wide range of excitation and emission spectra (Shaner *et al.*, 2005). Depending on the nature of the experiment one can choose to use a stable one that accumulates over time, which is especially useful when you are interested in the complete expression, or an unstable one which degrades as it ages and as such provides a snapshot of current expression levels in the cell.

One of the most commonly used fluorescent reporters today is the yellow fluorescent protein (YFP), which is originally a mutant of one of the first commercial fluorescent markers GFP, originating from the *Aequorea victoria* jellyfish (Tsien, 1998). YFP has a characteristic excitation peak at 514 nm, which experimentally is typically provided by a green argon laser, and emits light with a maximum at 527 nm (Tsien, 1998). These characteristics are especially valuable when performing fluorescence studies in cyanobacteria due to emission interference from native fluorophores (Sheen *et al.*, 1995).

When studying fluorescent behavior in photosynthesising organisms, the main problem in experiment design is to counter the effects of the inherent cell autofluorescence. This autofluorescent effect is caused by natural emission in the light absorbing chlorophylls of photosystem II (Sheen *et al.*, 1995). Important to note is that mature heterocysts lack photosystem II and as such do not autofluoresce. The chlorophylls in question absorb light, to at least some extent, over the full visual spectra as well as the upper end of the ultraviolet one and exhibit fluorescence between roughly 600 and 700 nm (Sheen *et al.*, 1995). Absorbance of the system does however dip considerably between 500 and 600 nm, placing the YFP excitation maximum at 527nm right in the perfect spot. In comparison, the excitation peaks of GFP is located at 395 and 475 nm which coincide with high absorption in chlorophyll a and b respectively, (figure 2) causing overexposure of autofluorescence using most detection techniques if GFP levels are not particularly strong.

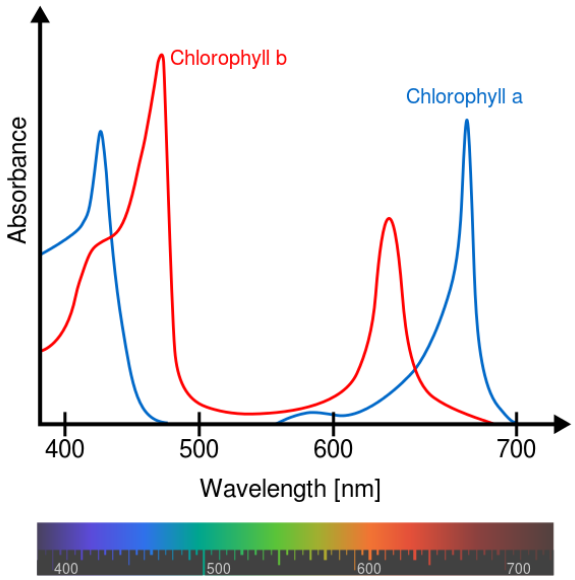


Figure 2: Typical absorption spectra of chlorophylls a and b spanning the visual wavelengths (Wikimedia commons, 2016)

One of the most efficient ways of detecting *in vivo* fluorescence is with the use of confocal fluorescence microscopy. The method has the possibility to provide clear pictures of individual cells in a filament with detection of several wavelengths of fluorescence simultaneously. With the use of multiple detectors and filters, specific for different wavelengths of light, it is possible to monitor YFP- and autofluorescence together and as such provide an image where you can clearly see which cells in a filament that are expressing the reporter. This is very useful when examining cell-specific expression during early stages of maturation, since at this point no morphological changes are present and autofluorescence has yet to fully decay. Proper handling of expression enhancement *in silico* also makes it possible to compare expression levels in individual cells even at very low expression rates.

The method itself is a further development of the conventional wide field version where fluorophores in a sample are excited by monochromatic laser light, causing them to fluoresce and return detectable emission wavelengths (Carlsson *et al.*, 1985).The main difference between the two methods is the introduction of a secondary pinhole close to the detector in the confocal version, which helps shut out non-specifically focused fluorescence (see figure 3).

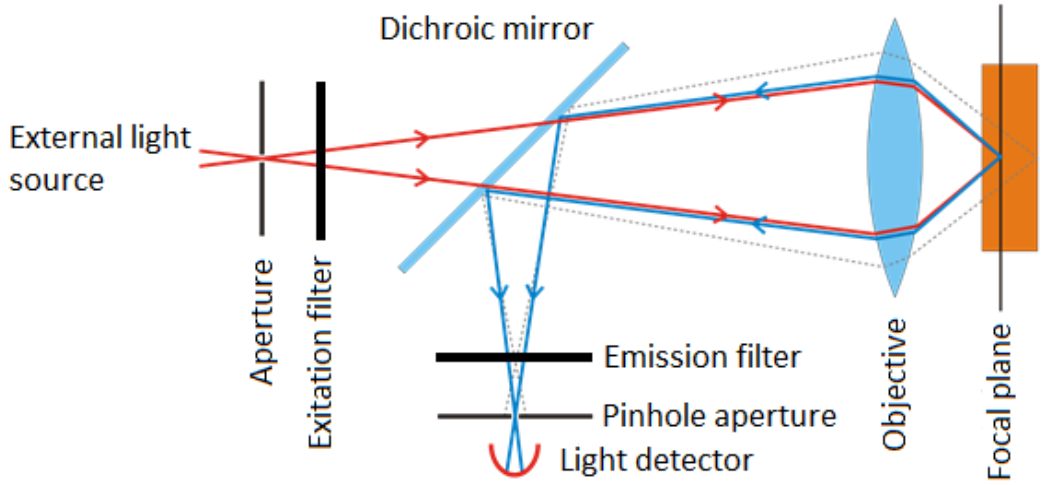


Figure 3: The basic outline of a confocal microscope. Light (shown as red) is emitted from an external light source, for example a strong fluorescence lamp or a laser, and passes through an adjustable aperture that regulates the areal light exposure, thus removing unwanted background light. The light then continues through an excitation filter, which removes all unwanted wavelengths, and a dichroic mirror before continuing through the objective and hitting the specimen where it gets absorbed by fluorophores. Fluorescence (shown as blue) is then emitted from the sample and passes back through the objective to the mirror where it is reflected through an emission filter. Depending on the focal plane originally hit by the specific beam of light, reflection will come at different angles making it possible to shut out unwanted planes by limiting the size of a second aperture, known as the pinhole, which is placed in front of the detector. This method prevents background fluorescence in pictures and provides sharper imaging than conventional wide field fluorescence microscopy (Carlsson *et al.*, 1985)(Wikimedia commons, 2016).

2. Results

2.1 Experimental outline

In order to study the importance for heterocyst-specific expression of each transcriptional regulated element of the NsiR I promoter and create as complete results as possible by including a negative control, six separate genetic constructs were created. The particular promoter constructs all followed a similar general outline as described in figure 4 and feature the differences described in table 1. From this point on, all strains will be referred to as described in table 1. The full sequences of each constructs are available in appendix C.

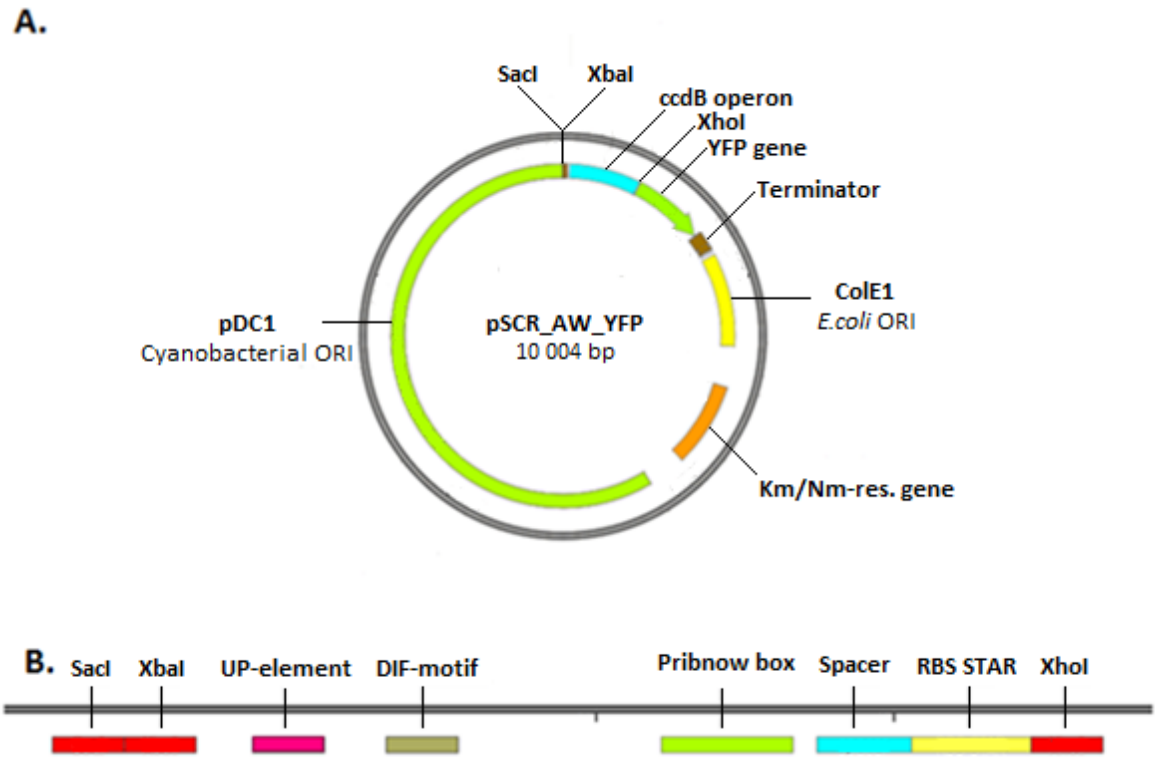


Figure 4: Schematic description of the pSCR_AW_YFP (pAW) plasmid with all its included elements (A), supplemented with a description of the main outline of all constructs used in this particular project (B). The constructs were introduced through traditional restriction based cloning, utilizing the SacI and XhoI sites, to replace the present ccdB operon (Cyan in A), a toxin-antitoxin system from *Escherichia coli*. The plasmid additionally features an YFP reporter gene (short light green segment in A) downstream of the ccdB region which is under regulation of the introduced promoter construct.

Table 1: Description of the differences in the examined genetic promoter constructs with regard to the important differing regions. Native refers to the element being present as found in the consensus sequence of the NsiR1 promoter repeats of *Anabaena sp*. 7120, DIF-motif and consensus relates to the consensus regions of heterocyst-specific promoters described by (Mitschke *et al.*, 2011) and scrambled means that the region consist of scrambled bases from the native consensus version.

	UP element	-35 Region	Spacer	-10 Region
Negative control	Not present	Not present	Not present	Not present
Synthetic	Not present	DIF-motif	Scrambled	Consensus
Native NsiR I (core)	Not present	DIF-motif	Native	Native
Native NsiR I (long)	Present	DIF-motif	Native	Native
Synthetic mix	Present	DIF-motif	Scrambled	Consensus
-10 change	Not present	DIF-motif	Native	P_{trc} -10

The first of our investigated promoter versions is a fully synthetic one, created according to suggestions in (Mitschke *et al.*, 2011). This promoter features theoretically optimized -10 and -35 regions based on the weblogo study (Appendix A). Remaining regions consist of scrambled versions of the consensus sequence of the twelve native promoter repeats as well as restriction sites for introduction into the vector and a ribosome binding site (RBS star). The reason for the scrambling of the non-conserved sites is to make sure that these do not impact the spatial situation of the expression.

The second and third promoters are both versions of the consensus sequence of the twelve native NsiR1-promoterrepeats of Anabaena sp. 7120, with addition of the necessary elements for cloning and expression mentioned above. The difference between the two of them is that the third promoter features an additional 25bp AT-rich region further upstream from the -35 site. If the DIF site does attract a sigma factor, this AT-rich region could serve as an UP-element, enhancing RNA polymerase binding.

The fourth and fifth promoters are essentially native/synthetic hybrids. The first of these two simply consists of the fully synthetic promoter with the addition of the AT-rich UP-site of the third promoter. Lastly, the fifth promoter features the sequence of the native core promoter but with a different - 10 site. In this particular one we have chosen to instead incorporate the -10 site of the well-studied constitutive non-heterocyst-specific trc10 promoter of *E. coli* (Huang *et al.*, 2010).

There are a number of questions we hope to be able to answer using these constructs:

1. Can the DIF motif be expressed outside of the heterocyst or is it completely heterocyst exclusive? This question can be answered by observing the expression pattern of any of the above promoters. If either show fluorescence in vegetative cells we can conclude that it is not so, otherwise results will point towards an exclusive behavior.

2. Is the DIF motive the sole requirement for heterocyst-specific expression? We can answer this question through a combination of assumptions based on the results of the YFP expression. In particular we will investigate the effects of the fifth promoter which features a non-heterocyst-specific -10 region. If this promoter provides YFP expression exclusively in heterocysts, we can confirm that the DIF motive is by itself capable to shift the spatial expression pattern of a promoter.

3. Will presence of the UP element increase expression levels of the YFP gene and does it affect the spatial expression pattern? This can be determined by investigation of the third and fourth promoters, which carry this region, compared to the first and second.

Finally, if all of the synthetically based promoters were to fail, we can conclude that the optimized regions fail to fulfill their purpose or that the non-conserved regions of the native version are of importance to where they are expressed. As such we would have further information to design future investigations.

2.2 Cloning strategy

All cloning in these experiments was performed utilizing restriction based cloning on commercially synthesized DNA oligonucleotides (Macrogen). The constructs were introduced into the plasmid through digestion of both the insert and the plasmid with the chosen restriction enzymes, SacI and XhoI, followed by ligation. Following this, the plasmid was transformed into DH5α *E. coli* via heat shock and positive colonies were selected for overnight on neomycin supplied Luria broth-agar plates. Presumed positive colonies were screened for, using colony PCR, and potential candidates grown in liquid culture overnight for plasmid preparation, which was then sent for sequencing (Macrogen).

Sequences were then analyzed using the online software MUSCLE (MUltiple Sequence Comparison by Log- Expectation) (Edgar, 2004) which lead to the discovery that the Plasmid contained an additional internal XhoI restriction site following the YFP gene that was not included in the plasmid map. As such, the first set of constructs all resulted in successful introduction of the desired promoter, at the loss of both the ccdB operon and the YFP gene. This error was handled in two separate ways. Firstly, site directed mutagenesis was performed on the internal restriction site of the plasmid with the purpose of deactivating it, allowing us to utilize the original strategy. However since this is a time consuming process in comparison, reintroduction of the YFP gene amplified from the original plasmid template was performed with the help of adjacent restriction sites. The secondary tactic proved successful for all constructs except the long version of the native NsiR I. As such, progress with this promoter lagged behind considerably and no results regarding confocal studies or quantitative fluorescence will be reported here.

Sequence confirmed constructs were then once again amplified by overnight culture before finally being introduced into wild type *N. punctiforme* through electroporation. All samples examined in this project were successfully transformed into the host strain.

2.3 Colony selection and culturing

The newly electroporated samples were first left to rest for 24 hours, either on cellulose membranes on ammonium supplied antibiotic free $BG11_0$ (Appendix B) agar plates or in ammonium supplied liquid $BG11_0$ media, before being transferred to neomycin containing plates for selection. The selection process lasted between 14-21 days depending on the thickness of the original culture and plates were changed once a week during the process to minimize risk of contaminations and maximize access to nutrients. When clear single cell colonies could be observed, these were transferred directly onto new antibiotic supplied plates to finalize the selection process.

Following this, selected colonies were transferred into 10 ml six-well plates containing liquid $BG11_0$ supplied with neomycin to mature into observable cultures, additional plates supplied with ammonium was also prepared to be able to compare between them. This process lasted for ~7 days at which point the cultures were examined by confocal fluorescence microscopy to determine the effects of the promoters on reporter protein expression in fully matured heterocyst containing filaments.

For further experimental purposes, all samples were lastly moved in duplicates into e-flasks containing 50 ml of $BG11_0$ supplied with ammonium and neomycin, which were resupplied with ammonium every other day (Figure 5). At full maturation, part of the cultures were also removed to create frozen stocks by spinning the culture down, removing the supernatant and adding 10% pure DMSO before storing in -80℃.

Figure 5: Picture showing the growth states of five out of six strains after one month of culturing. Left to right as described in table 1: negative control, -10 change, synthetic mix, synthetic, and native NsiR I core. The native NsiR I long was not transformed at this point in time.

2.4 Confocal fluorescence microscopy

To be able to visualize the expression of YFP in individual cells of the examined strains, confocal microscopy was used. The experiments were all performed with a similar experimental setup, as described in materials and method (4.17). Results from these experiments proved the successful creation of a fully functioning heterocyst-specific synthetic promoter as well as giving some insight into the expression behavior of the same *in vivo*. It is here also shown that the -10 region is definitely important to the cell specificity of the promoter and that expression behavior is similar in the promoters containing similar elements. Additionally, it was discovered that expression of the NsiR I promoter and the Up-element containing mixed synthetic promoter, usually occurs in a broader area of roughly five cells at early differentiation, before slowly contracting into a single differentiated heterocyst after roughly 24 hours. It was shown that the negative control, lacking promoter, showed no detectable fluorescence throughout any of the experiments regardless of ammonium being supplied or not, as such confirming the function of the other strains. During the process, a new experimental method for studying single stationary filaments over time described in material and method (4.17) was also developed with the purpose of creating a differentiation timeline. Judging from the results presented here, all of the promoters examined during these tests seem to provide reporter expression during at least 48 hours of growth under nitrogen fixing conditions, without any visibly detectable drop in activity.

2.4.1 Initial trials

Initial trials with the confocal microscope were all conducted in liquid culture and proved that all of the constructed strains were fully functional. It was clear to see that the control was completely silent and that expected cell specificity could be observed in the native core promoter as well as in the two synthetic constructs. The switch of the native -10 region to that of P_{trc} did however prove to have critical effect on the behavior of the promoter and rendered in a constitutive behavior even in vegetative cells. All of the nitrogen grown constructs did at this point show some evidence of heterocyst development which was attributed to depletion of nitrogen in the media. As such rigorous efforts to prevent this in further experiments was employed.

2.4.1.1 Negative control

Neither the ammonium grown nor the nitrogen fixing control samples showed any traces of YFP fluorescence, as was to be expected. From the pictures we can conclude that this construct efficiently terminates any possible leakage of the system. The bright field microscopy filter of the upper right image was not included due to a system malfunction; however this does not impact the results (figure 6).

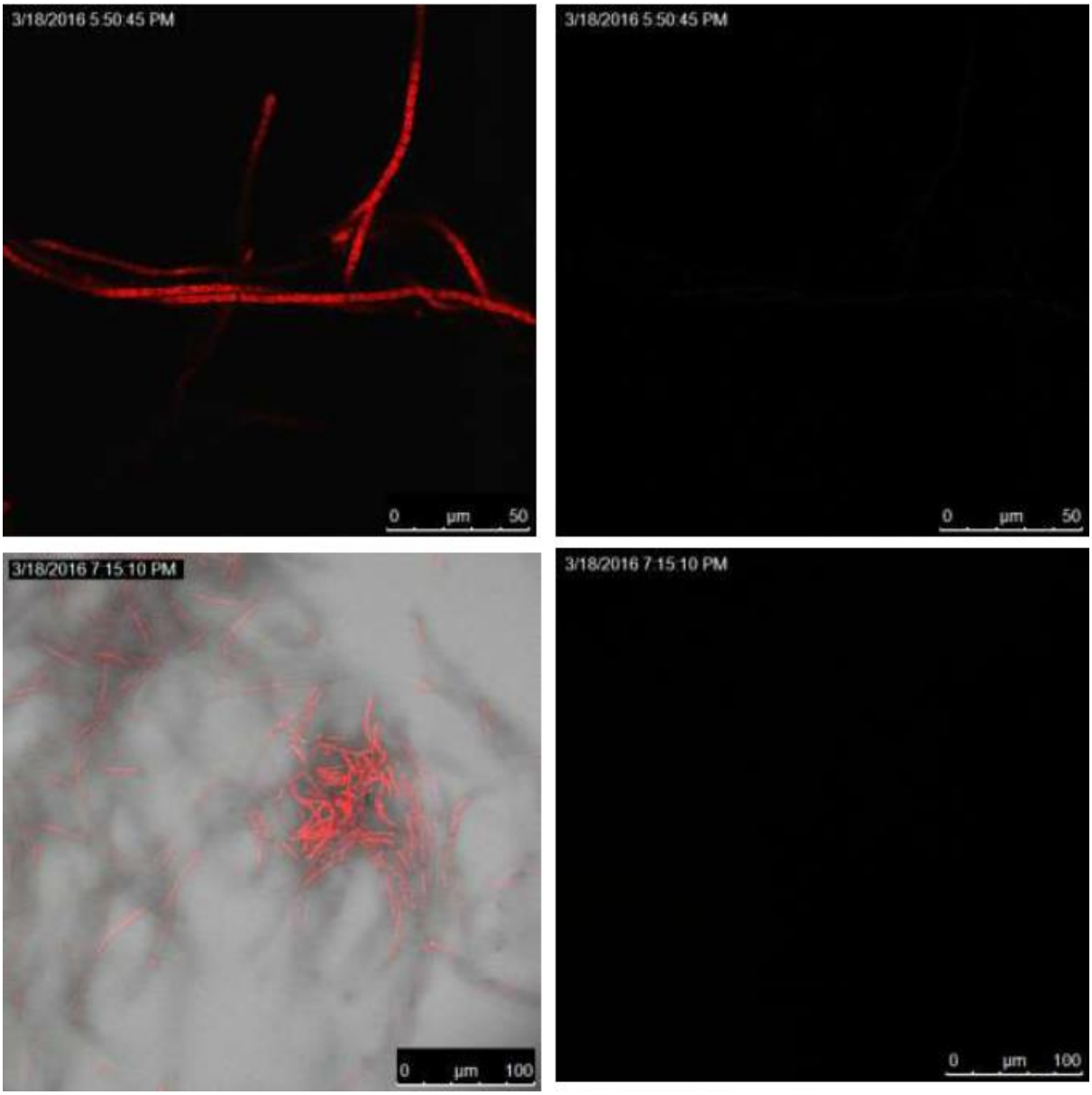


Figure 6: Confocal microscopy pictures depicting the fluorescence of YFP from a non-promoter containing control plasmid in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture lacking bright field filter (Ammonium grown), light microscopy filter image left out. Top right: YFP-fluorescence (ammonium grown). Bottom left: Composite picture (nitrogen fixing). Bottom right: YFP-fluorescence (nitrogen fixing).

2.4.1.2 Synthetic promoter

The pictures from the synthetic promoter constructs gave mixed results. One of the duplicates (not displayed here) showed no fluorescence at all and was disregarded as a false positive. This reasoning is strengthened by it also being the only surviving colony from a selection plate. As such an additional colony was picked from another plate for future experiments. The results obtained from the functioning sample did however show fluorescence in a number of cells, of which some also morphologically resembled heterocysts. In this sample we also see a clear increase in amount of fluorescent cells when grown under nitrogen fixing conditions. Due to difficulties in finding single filaments it is very hard to determine whether the YFP-fluorescing cells also auto-fluoresce or not which could be used to indicate whether they are true heterocysts or not (figure 7).

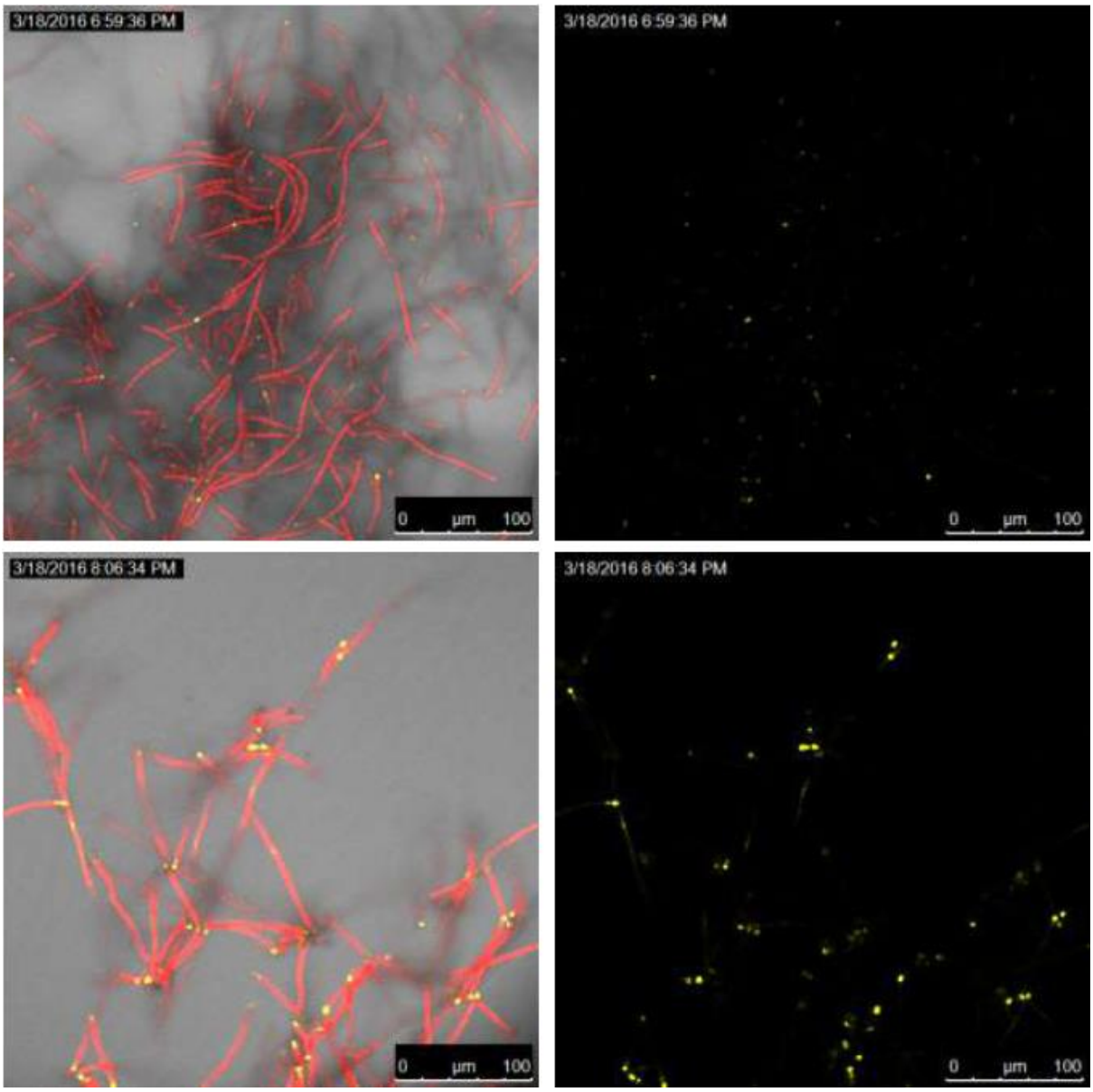


Figure 7: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture (Ammonium grown). Top right: YFP-fluorescence (ammonium grown). Bottom left: Composite picture (nitrogen fixing). Bottom right: YFP-fluorescence (nitrogen fixing).

2.4.1.3 Native NsiR I core promoter

The preliminary images of the constructs containing the short native promoter show clear YFP expression in a number of cells; however they are still being vastly outnumbered by the auto-fluorescent vegetative cells. The picture does show a clear preference of fluorescence towards the end of fragments which would indicate these cells to be heterocysts. This can be explained by the fact that the samples where homogenized with a syringe prior to microscopy and the filaments often break close to these cells. In addition we can conclude that fluorescent cells can be found in the ammonium grown cells as well, indicating that these at some point ran out of ammonium during growth and started producing heterocysts. The problem with finding single filaments persisted in this sample but the results are regardless a clear indication towards heterocyst-specific expression (figure 8).

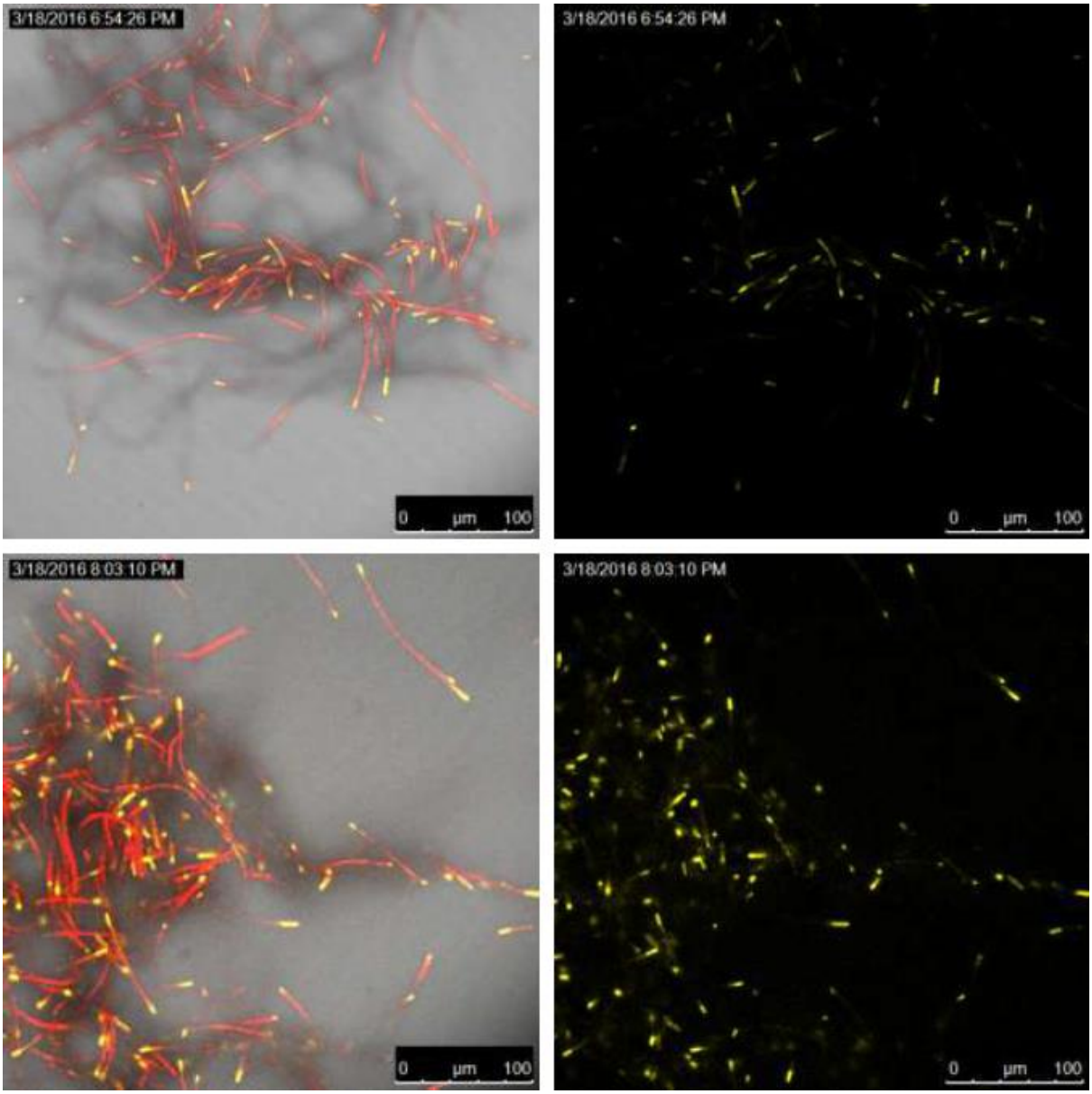


Figure 8: Confocal microscopy pictures depicting the fluorescence of YFP produced using the short native NsiR core promoter of *Anabaena sp*. in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture (ammonium grown). Top right: YFP-fluorescence (ammonium grown). Bottom left: Composite picture (nitrogen fixing). Bottom right: YFP-fluorescence (nitrogen fixing).

2.4.1.4 Synthetic mix promoter

The Mix construct provided similar results to the fully synthetic one (figure 9), which is to be expected since the only difference is in the feature of an AT-rich upstream region. In other words it should behave exactly the same when YFP is allowed to accumulate. The only noteworthy difference between the two strains is that both samples in the duplicate for this construct were viable for investigation, which further strengthens the assumption regarding the false positive in the synthetic sample.

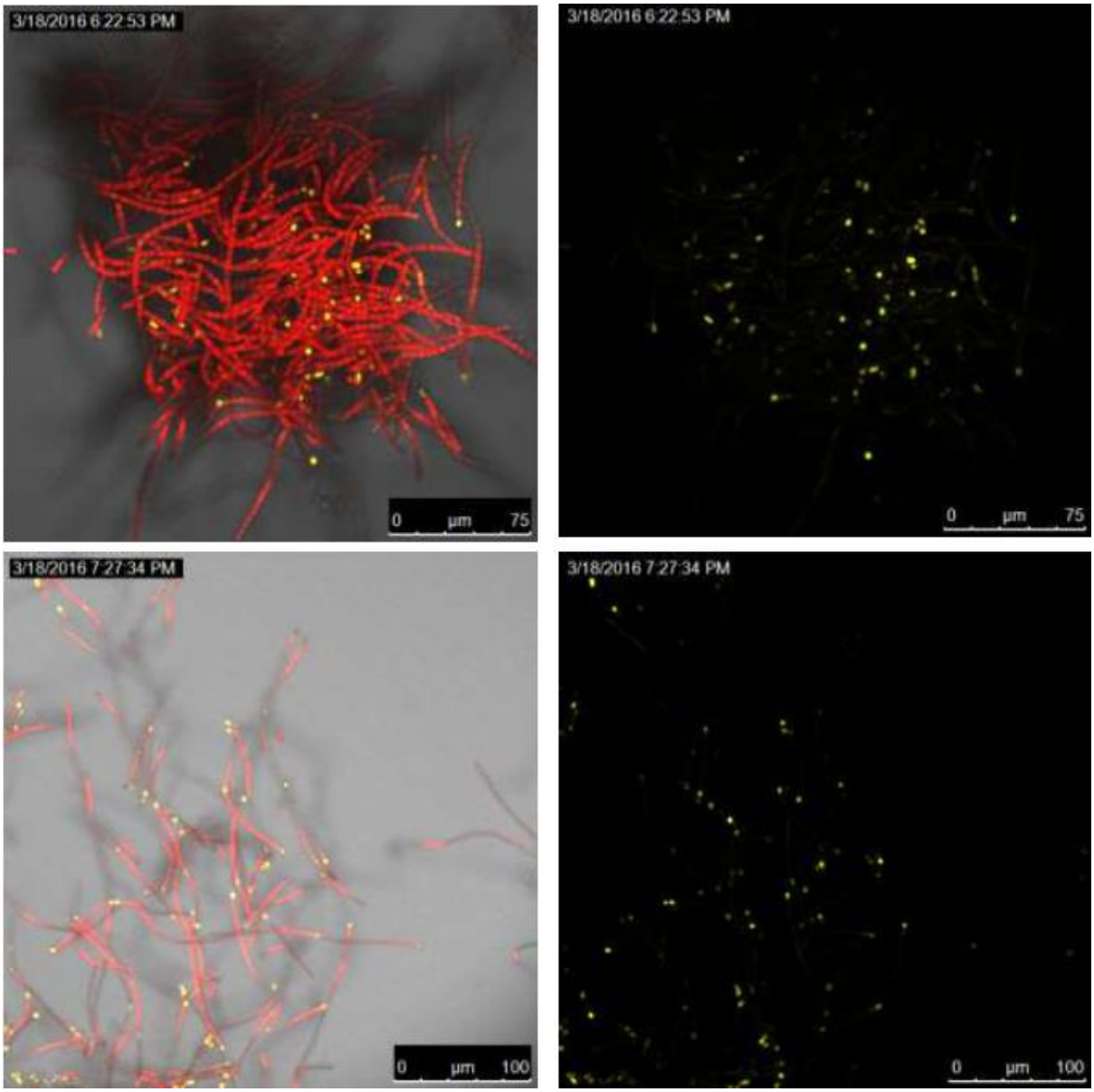


Figure 9: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter featuring a conserved AT-rich UP site in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture (ammonium grown). Top right: YFP-fluorescence (ammonium grown). Bottom left: Composite picture (nitrogen fixing). Bottom right: YFP-fluorescence (nitrogen fixing).

2.4.1.5 -10 change promoter

The -10 change promoter samples clearly stick out when compared to the others. We can clearly see an over expression of YFP throughout all of the cells, regardless of being supplied with ammonium or not, (figure 10) which in turn confirms that, at least in this system, the pribnow-box is interesting for heterocyst-specificity. Additionally this serves as a positive control which clearly indicates specificity in the expression shown across the other samples.

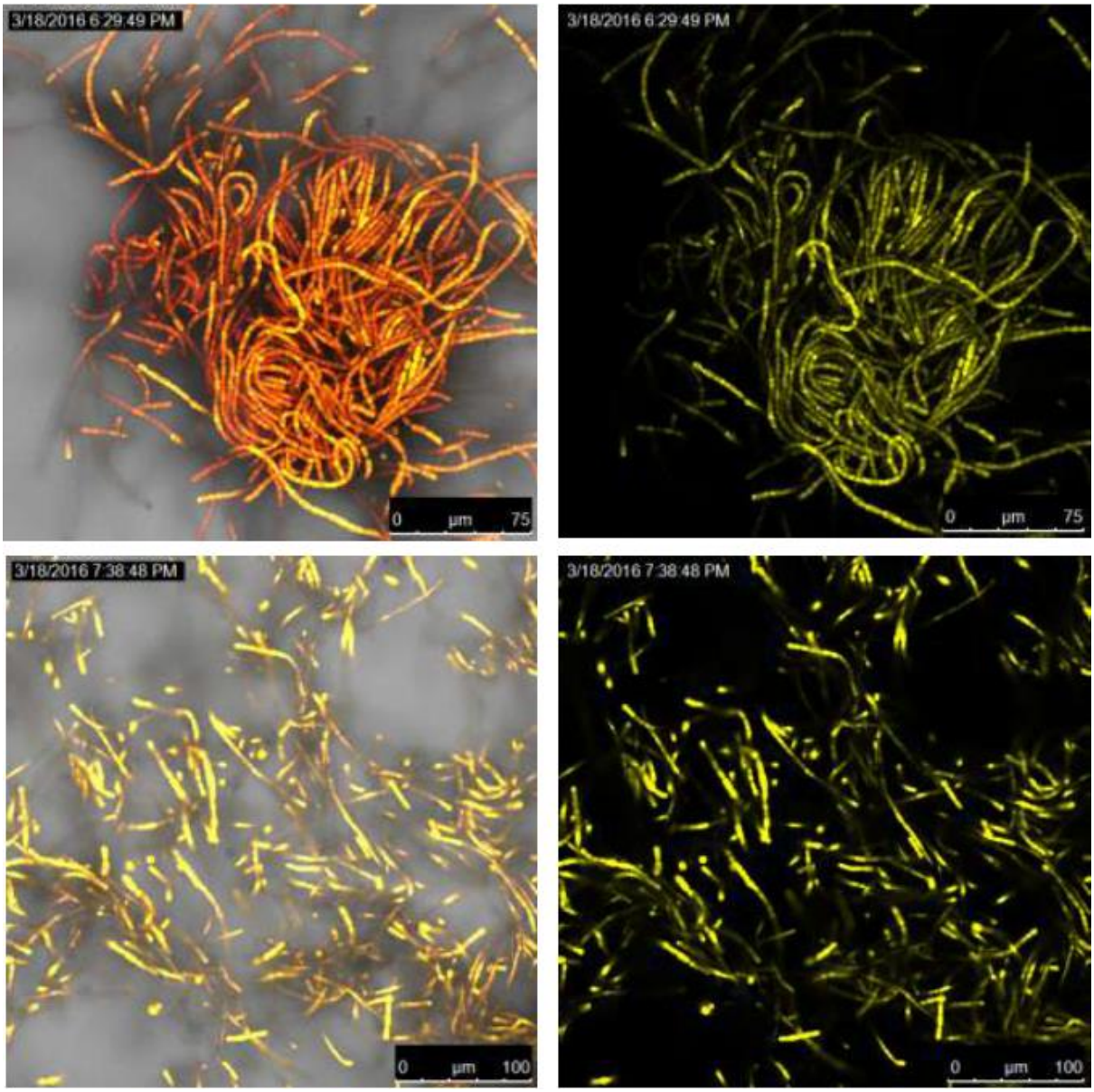


Figure 10: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter featuring the -10 region of P_{trc} in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture (ammonium grown). Top right: YFP-fluorescence (ammonium grown). Bottom left: Composite picture (nitrogen fixing). Bottom right: YFP-fluorescence (nitrogen fixing).

2.4.2 Six hour liquid culture trial

After cell specific expression was established in most of the constructs, experiments proceeded with temporal investigations over 6 hours of nitrogen deprivation. Sampling was performed every 1.5 hours, to investigate how the expression changes over time. An additional sample was taken after 24 hours to be used as a reference. Due to the very small variations over the first six hours only three time points (0, 6, and 24 hours) were elected for display in these results. A nitrogen free environment was established through nitrogen step down as described in materials and method (4.26) and the samples were observed on a thin slice of agar gel with the purpose of minimizing the movement of the liquid in the sample as well as prevent the stacking of filaments. The behavior of the negative control was already considered clear at this point and was left out of this experiment.

These experiments proved an inherent leakage in all of the cell-specific constructs, as background fluorescence was clearly detectable. Further, the 24 hour controls proved heterocyst-specific expression. It is impossible to determine if the leakage is caused by the characteristics of the promoters themselves or the system. Additionally, the experiment showed that high YFP fluorescence could spill over into the autofluorescence detection spectra and as such create clear expression in heterocyst cells that would otherwise lack this fluorescence (figure 11). A difference in the behavior was detected regarding the mixed synthetic promoter compared to the synthetic and short core. The mixed strain showed several brightly fluorescent cells a short distance from each other, a behavior which was later attributed to a more advanced developmental stage. Finally, it was discovered that 6 hours was not enough to clearly distinguish which cells were to become heterocysts using this method due to interference from the background fluorescence. The desire to be able to investigate the same filament over time was also arisen during this investigation since it was impossible to make a just comparison. This once again led to the development of a new methodical setup for the following microscopy experiments.

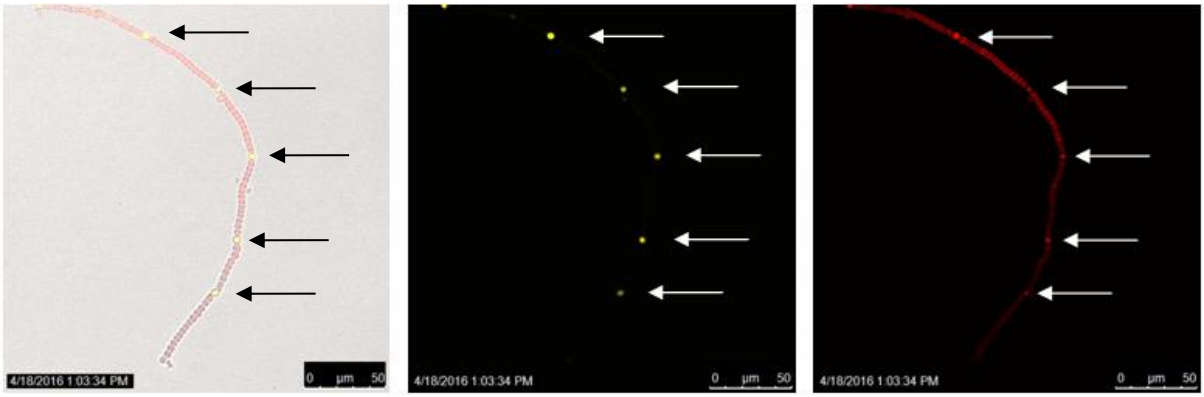


Figure 11: Confocal microscopy pictures depicting the fluorescence of YFP produced using the short native NsiR promoter of *Anabaena sp.* in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Arrows show where fluorescence spillover into the autofluorescence uptake. Pictures were taken 24 hours after nitrogen deprivation.

2.4.2.1 Synthetic promoter

The first image of the nitrogen-deprived synthetic promoter strain, taken at zero hours, shows a slight homogenous background emission caused by unspecific expression (figure 12). The cause of this is unknown but it could either be attributed to the behavior of the promoter or the expression system. It is however possible to distinguish some regions with slightly higher fluorescence intensity that could potentially be budding pro-heterocysts (prospective heterocyst) or otherwise enhanced regions. It is noteworthy that the expression at this point needed considerable *in silico* enhancement to become visible.

After six hours of nitrogen deprivation, we can clearly see a terminal pre-heterocyst (predetermined heterocyst), which exhibits morphological differences compared to surrounding cells, at the top of the picture as well as some budding ones towards the middle and lower part. For all other pictures up until this point, it was not possible to clearly determine anything more than plausible regions of heterocyst development.

Observing the final 24 hour control picture, we see three very clear mature heterocysts situated at expected distance from each other. Fluorescence intensity now also vastly outmatches that of the background bringing a clear expression pattern.

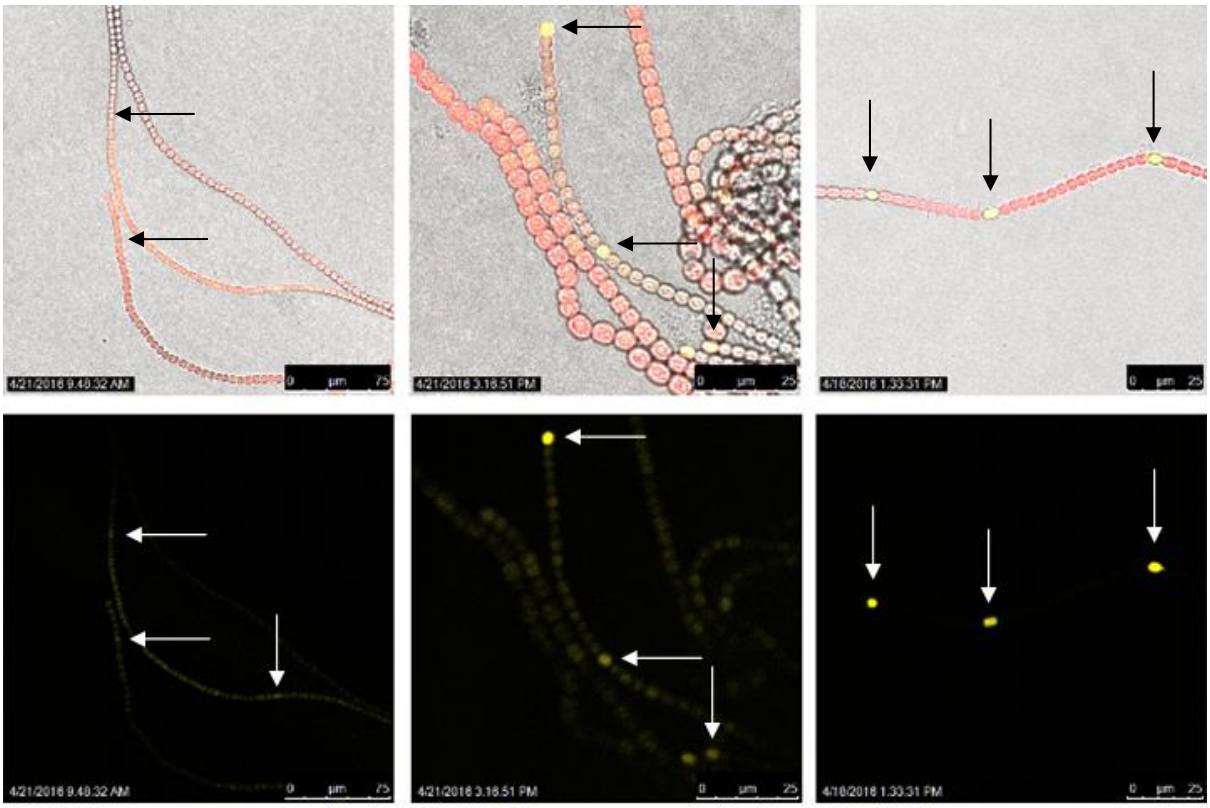


Figure 12: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture at time point zero after nitrogen deprivation. Bottom left: YFP-fluorescence at time point zero. Top middle: Composite picture six hours after nitrogen deprivation. Bottom middle: YFP-fluorescence after six hours. Top right: Composite picture 24 hours after nitrogen deprivation. Bottom right: YFP- fluorescence after 24 hours. Arrows show presumptive heterocysts, pre-heterocysts and pro-heterocysts.

2.4.2.2 Native NsiR I core promoter

The strain containing the native core promoter construct displayed a lot more background fluorescence compared to the synthetic one. This made identification of potential heterocyst candidates a lot harder since differences were not clear. Relying on the decreased levels of autofluorescence in pre-heterocysts was not reliable either do to the observed spillover effect (figure 11.) The fact that the composite pictures appear yellow in a similar fashion as the -10 change construct from the initial investigation is however just an artifact of *in silico* enhancement, the autofluorescence is still stronger in intensity (figure 13).

Even after six hours of differentiation, background fluorescence was still too high to accurately spot any true heterocyst maturation. As such, purposed pre-heterocysts in the early stages are strictly based on the immediate surroundings and morphology (figure 13).

After 24 hours we do however observe the clear differentiation pattern that was expected with regularly spaced heterocysts.

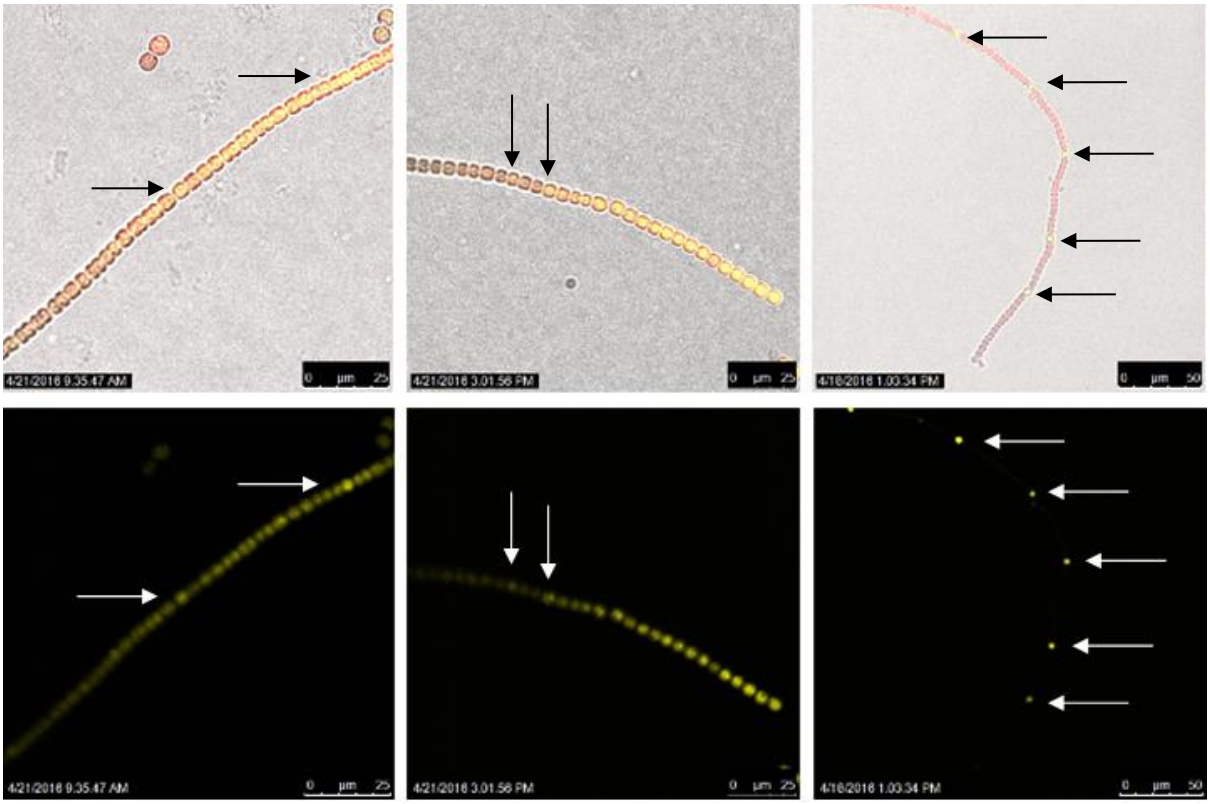


Figure 13: Confocal microscopy pictures depicting the fluorescence of YFP produced using the short native NsiR I core promoter of *Anabaena sp*. in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture at time point zero after nitrogen deprivation. Bottom left: YFP-fluorescence at time point zero. Top middle: Composite picture six hours after nitrogen deprivation. Bottom middle: YFP-fluorescence after six hours. Top right: Composite picture 24 hours after nitrogen deprivation. Bottom right: YFP- fluorescence after 24 hours. Arrows show presumptive heterocysts, pre-heterocysts and pro-heterocysts.

2.4.2.3 Synthetic mix promoter

The synthetic mix promoter provided the most interesting change in expression of the examined strains throughout all of the strains in this experiment. Already at zero hours we can clearly see cells with increased YFP expression levels roughly interspaced by 3-5 non-fluorescing cells. Even though this strain also features considerable background levels, these cells are easily distinguishable (figure 14).

After six hours of differentiation, some cells have further increased in fluorescence intensity and regions of three or more cells with a slight increase in comparison with the background can be observed.

However, after complete differentiation at 24 hours, the previous behavior with multiple fluorescent cells situated close to each other has disappeared and one can once again observe the expected normal behavior with heterocyst-specific expression at roughly every tenth position.

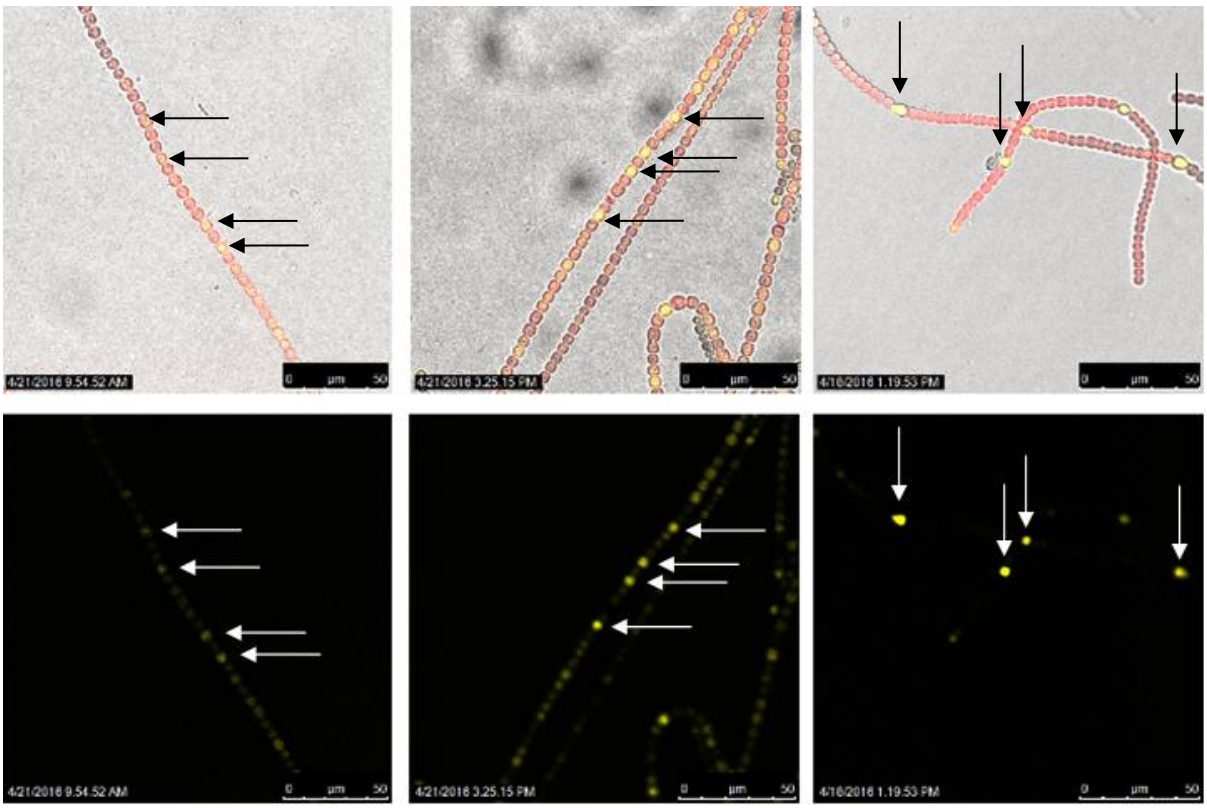


Figure 14: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter featuring a conserved AT-rich UP site in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture at time point zero after nitrogen deprivation. Bottom left: YFP-fluorescence at time point zero. Top middle: Composite picture six hours after nitrogen deprivation. Bottom middle: YFP-fluorescence after six hours. Top right: Composite picture 24 hours after nitrogen deprivation. Bottom right: YFP- fluorescence after 24 hours. Arrows show presumptive heterocysts, pre-heterocysts and pro-heterocysts.

2.4.2.4 -10 change promoter

The -10 change promoter strain was simply included in this experiment as a positive control. Using these pictures as a reference tool it is possible to determine if a region in another sample should be deemed having increased fluorescence or if it is simply due to natural variation in the filament. As seen at both 0 hour and 6 hours (figure 15), there are some variations present even in these samples, indicating that the slight shifts seen at zero hours in the synthetic (figure 12) and NsiR I core (figure 13) might simply be natural variations in YFP expression rather than actual shifts. The fully mature sample also shows us how clearly visible a true heterocyst is even when the full filament is fluorescing.

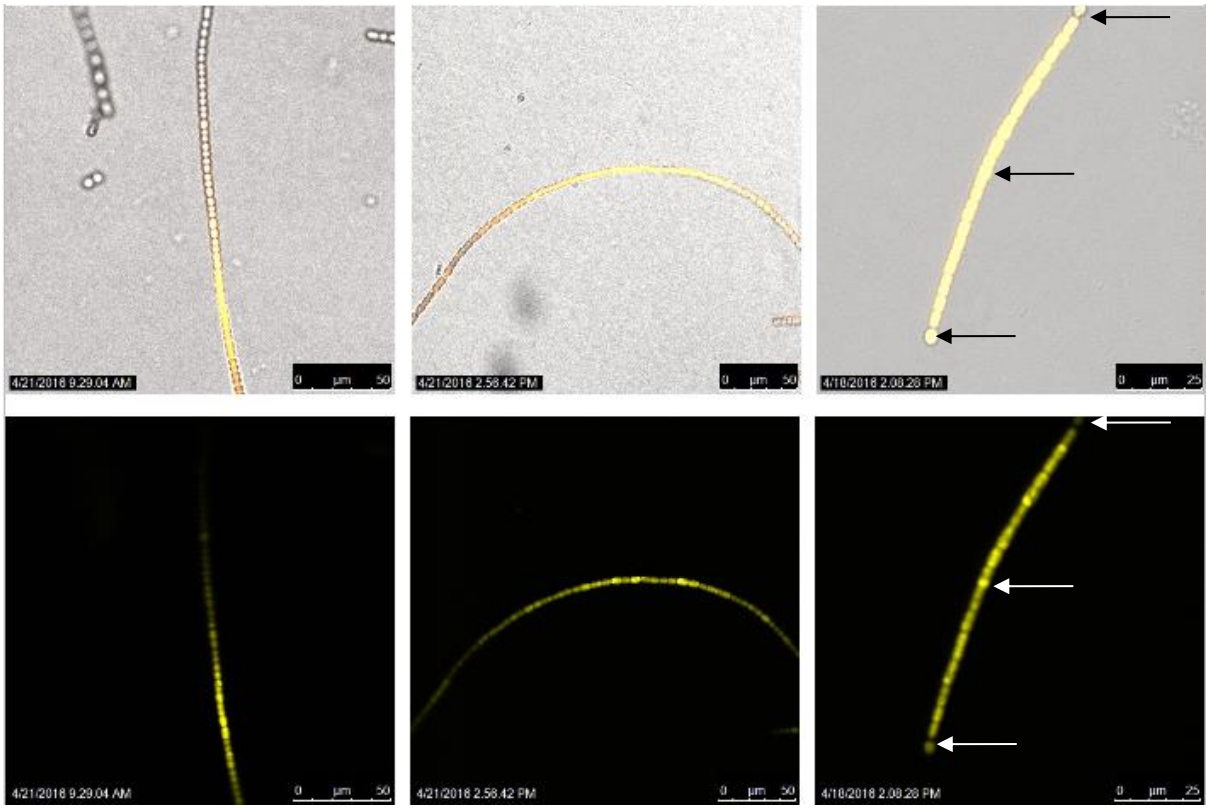


Figure 15: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter featuring the -10 region of P_{trc} in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Top left: Composite picture at time point zero after nitrogen deprivation. Bottom left: YFP-fluorescence at time point zero. Top middle: Composite picture six hours after nitrogen deprivation. Bottom middle: YFP-fluorescence after six hours. Top right: Composite picture 24 hours after nitrogen deprivation. Bottom right: YFP- fluorescence after 24 hours. Arrows show presumptive heterocysts, pre-heterocysts and pro-heterocysts.

2.4.3 48 hour immobilized culture experiment

For the final confocal experiments, a new experimental tactic was developed to enable the study of single filaments over a time period of 48 hours (as described in material and method). Pictures in this experiment were captured with three hour intervals over the first twelve hours and then once at 24 and 48 hours after nitrogen deprivation.

In these experiments, the previously discovered pattern with areas of greater fluorescence at initial maturation was again observed for the NsiR I core and mixed synthetic strains. It was however not as clear for the core version which still displayed high levels of background fluorescence. As time passed, these areas gradually retracted to a few highly fluorescent cells and ended as one or two pre-heterocysts. The synthetic promoter showed completely cell specific expression throughout all of the differentiation process, but it is possible that this might be caused by the low fluorescence levels, making a lot of enhancement and as such possibly not correctly displaying the surrounding cells. No changes of the pattern could be observed in the -10 change strain or negative control throughout the experiments.

Some general observations regarding the behavior of the filaments were however noticed. Cell growth in this kind of immobilized environment seems to make the filaments bend in a zigzag and eventually break at the bends (figure 16) Additionally, it was noticed that some strongly fluorescent cells may still divide into new ones and then share the original fluorescence between them (figure 17). It seems that differentiation occurs at a much slower rate when cells are grown under these conditions rather than in liquid culture. It was not possible to see anything that could be considered a mature heterocyst until after 48 hours of immobilization, as compared to 24 for liquid grown (figure 18). And finally it is observed that terminal heterocysts are not necessarily caused by breakage of the filament, cells towards the end of a filament seems more likely to develop into heterocysts regardless (figure 19).

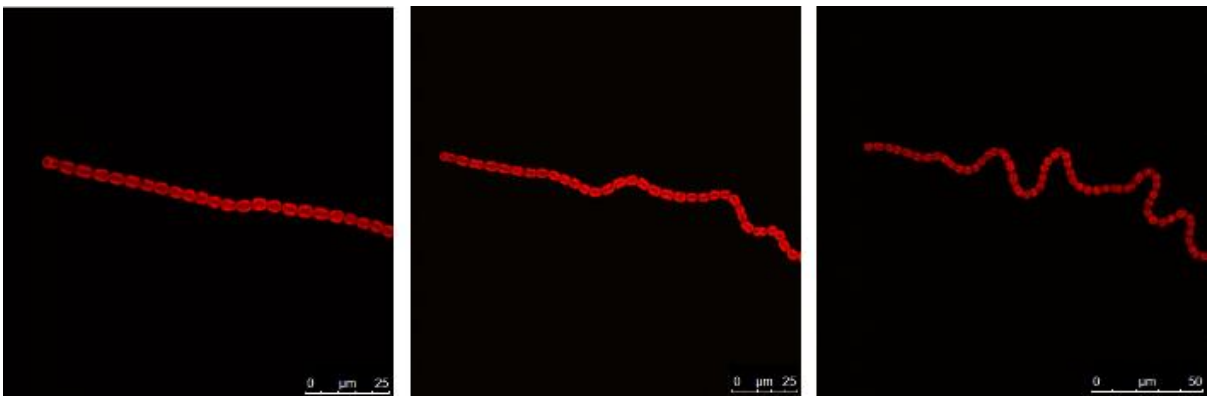


Figure 16: Confocal microscopy pictures depicting the fluorescence of YFP from a non-promoter-containing control plasmid in *Nostoc punctiforme*. PSII autofluorescence here shown as red. The picture shows how immobilized filaments on an agar gel bends as they grow. Left: three hours after immobilization. Middle: Nine hours after immobilization. Right: 24 hours after immobilization.

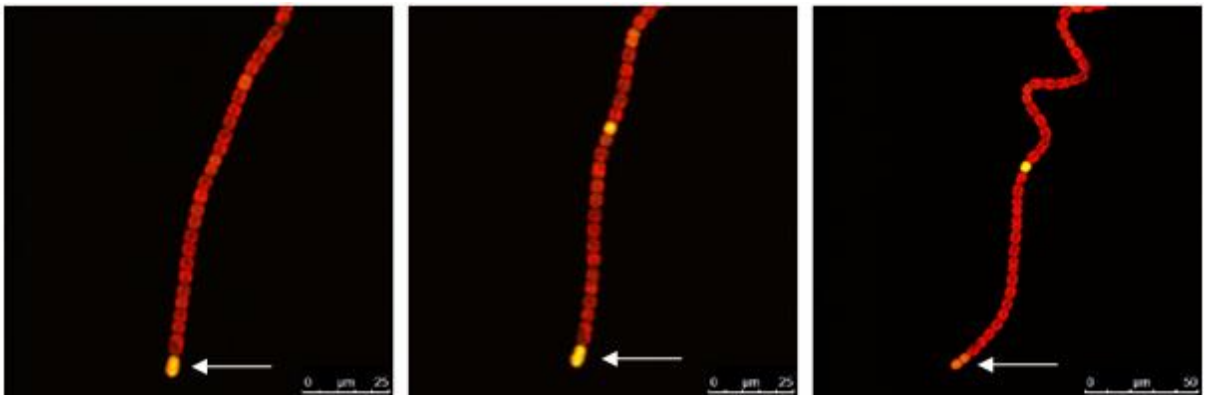


Figure 17: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. This picture shows the splitting of a highly fluorescent cell toward the end of the filament. Left: nine hours after immobilization. Middle: 12 hours after immobilization. Right: 24 hours after immobilization. Arrows mark the interesting cell.

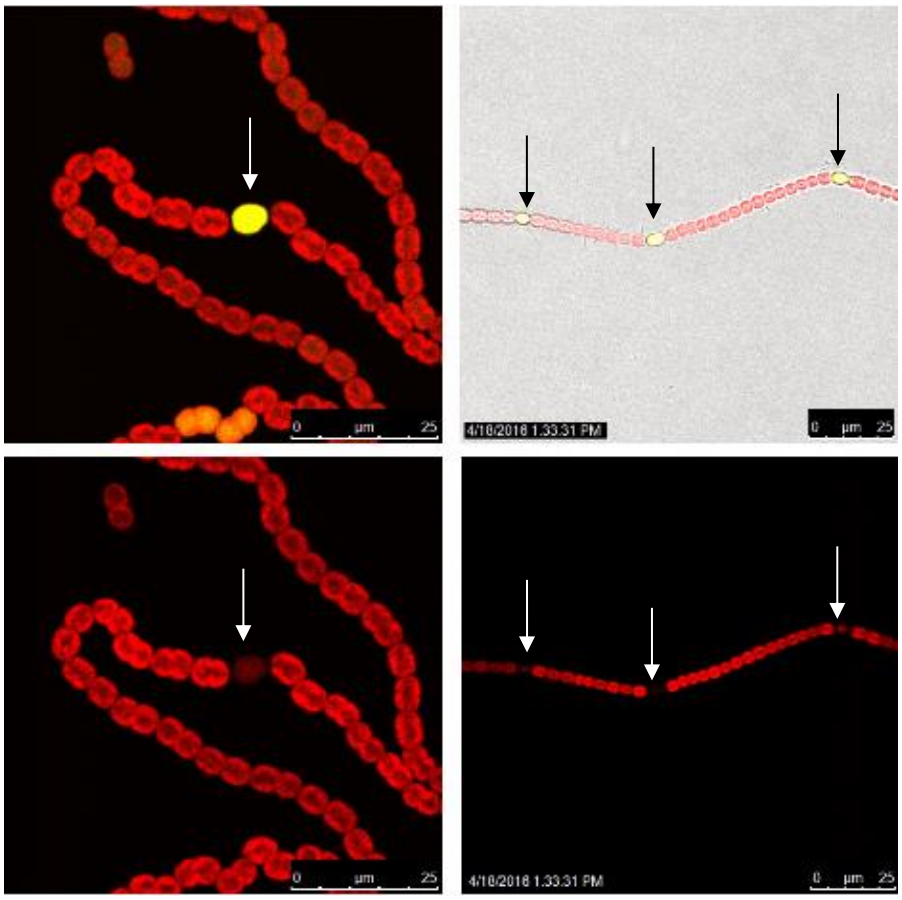


Figure 18: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. This picture shows the differences in heterocyst development between cells grown in liquid culture and cells immobilized on plates at different time points. Heterocysts can be identified by the decrease of auto-fluorescence and difference in morphology. Top left: composite picture taken 48 hours after immobilization. Top right: composite picture taken after 24 hours of differentiation in liquid media. Bottom left: picture of auto-fluorescence 48 hours after immobilization. Bottom right: picture of auto-fluorescence taken after 24 hours of differentiation in liquid media. Arrows mark the interesting cells.

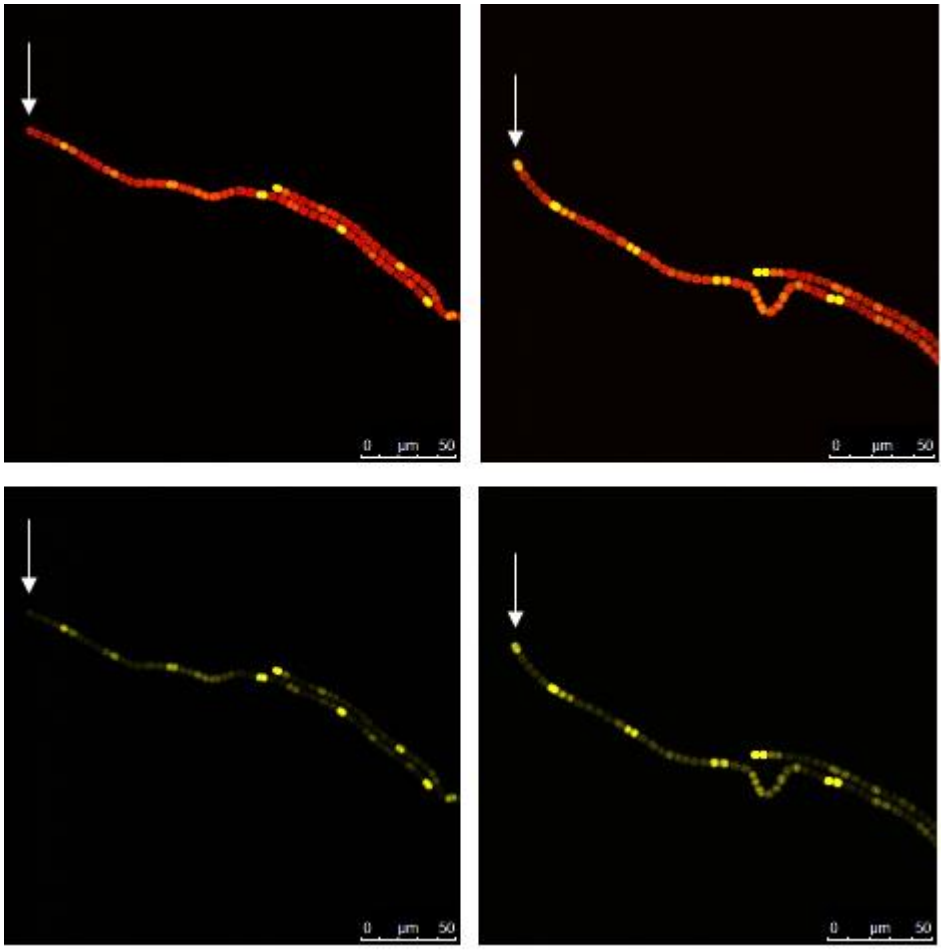


Figure 19: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter featuring the AT-rich upstream site of NsiR in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Arrows mark the interesting region. This picture shows how a terminal heterocyst is developed with time without any breakage of the filament. Top left: composite picture taken 24 hours after immobilization. Top right: composite picture taken 48 hours after immobilization. Bottom left: picture of YFP-fluorescence 24 hours after immobilization. Bottom right: picture of YFP-fluorescence 48 hours after immobilization.

2.4.3.1 Negative control

As in previous experiments, the control sample showed no signs of YFP-fluorescence. During growth of the filament it started curving at an early state and eventually ended up breaking into two pieces sometime between 24-28 hours (figure 20). An interesting observation is that no cells in the filament showed signs of losing their auto fluorescence after 48 hours indicating that no pro-heterocysts were present in this particular filament. The cause of this is unknown but it could very well be related to residual ammonium present in the cells in combination with the retarded growth rate in this specific method.

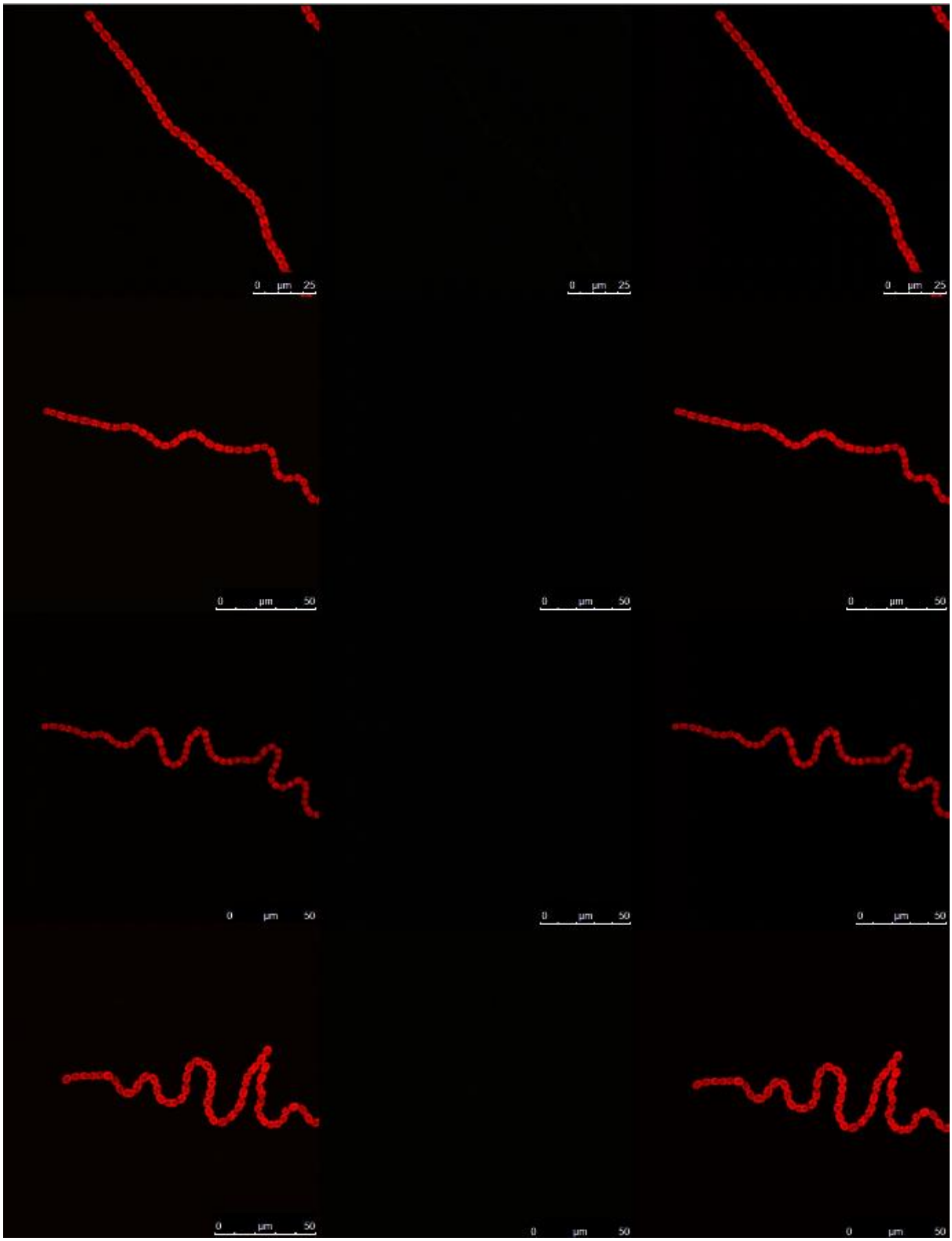


Figure 20: Confocal microscopy pictures depicting the fluorescence of YFP from a non-promoter containing control plasmid in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Left to right shows the composite picture, YFP fluorescence and PSII autofluorescence. Top to bottom shows pictures taken at 0, 12, 24 and 48 hours after immobilization.

2.4.3.2 Synthetic promoter

The strain containing the synthetic promoter showed very early signs of cell specific expression with two cells standing out in expression level already from zero hours after immobilization. These cells showed increased levels of YFP-fluorescence throughout the 48 hours, although the one located in the terminal end split into two after roughly 12 -24 hours. 48 hours into the experiment, it is possible to observe a slight decrease in the autofluorescence of the non-terminal cell as well as a non-fluorescent area surrounding it, indicating pre-heterocyst formation. In addition we also identify an increase of fluorescence in an area between the two previously fluorescent cells which might be a new differentiating heterocyst (figure 21). Similarly to the other constructs, the filament curved considerably.

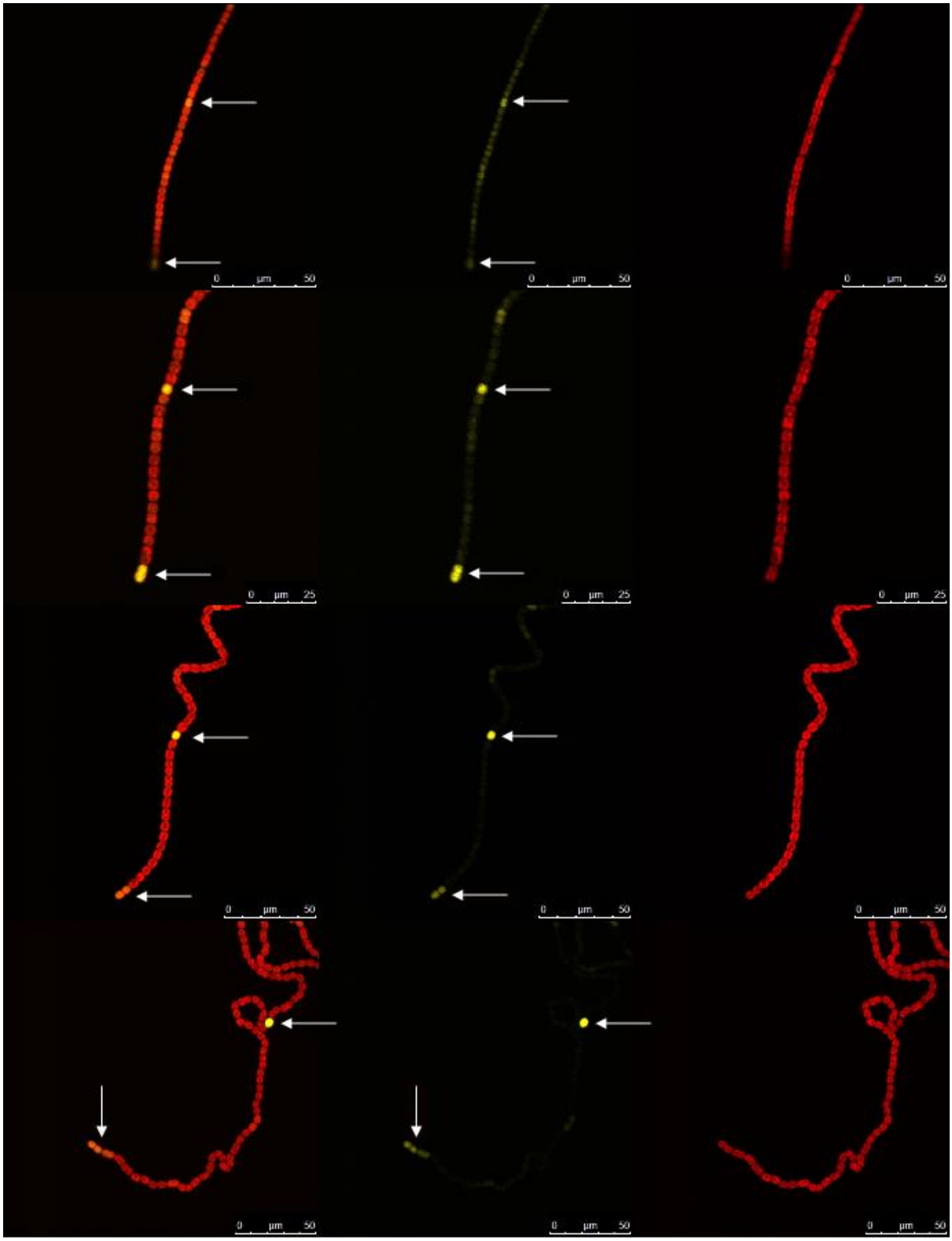


Figure 21: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter in *Nostoc punctiforme.* PSII autofluorescence here shown as red and YFP fluorescence as yellow. Left to right shows the composite picture, YFP fluorescence and PSII autofluorescence. Top to bottom shows pictures taken at 0, 12, 24 and 48 hours after immobilization. Regions of heterocyst development are here marked with arrows.

2.4.3.3 Native NsiR I core promoter

The native NsiR I core promoter proved to have a similar expression pattern as previously with very strong background fluorescence. Some regions showed stronger fluorescence from the beginning and did with time either develop into single fluorescent cells with a slight decrease in autofluorescence or stay as enhanced regions, as can be clearly seen in the upper terminal end of figure 22. Cell growth caused the original filament to break in two places and eventually caused a nested section of three separate filaments.

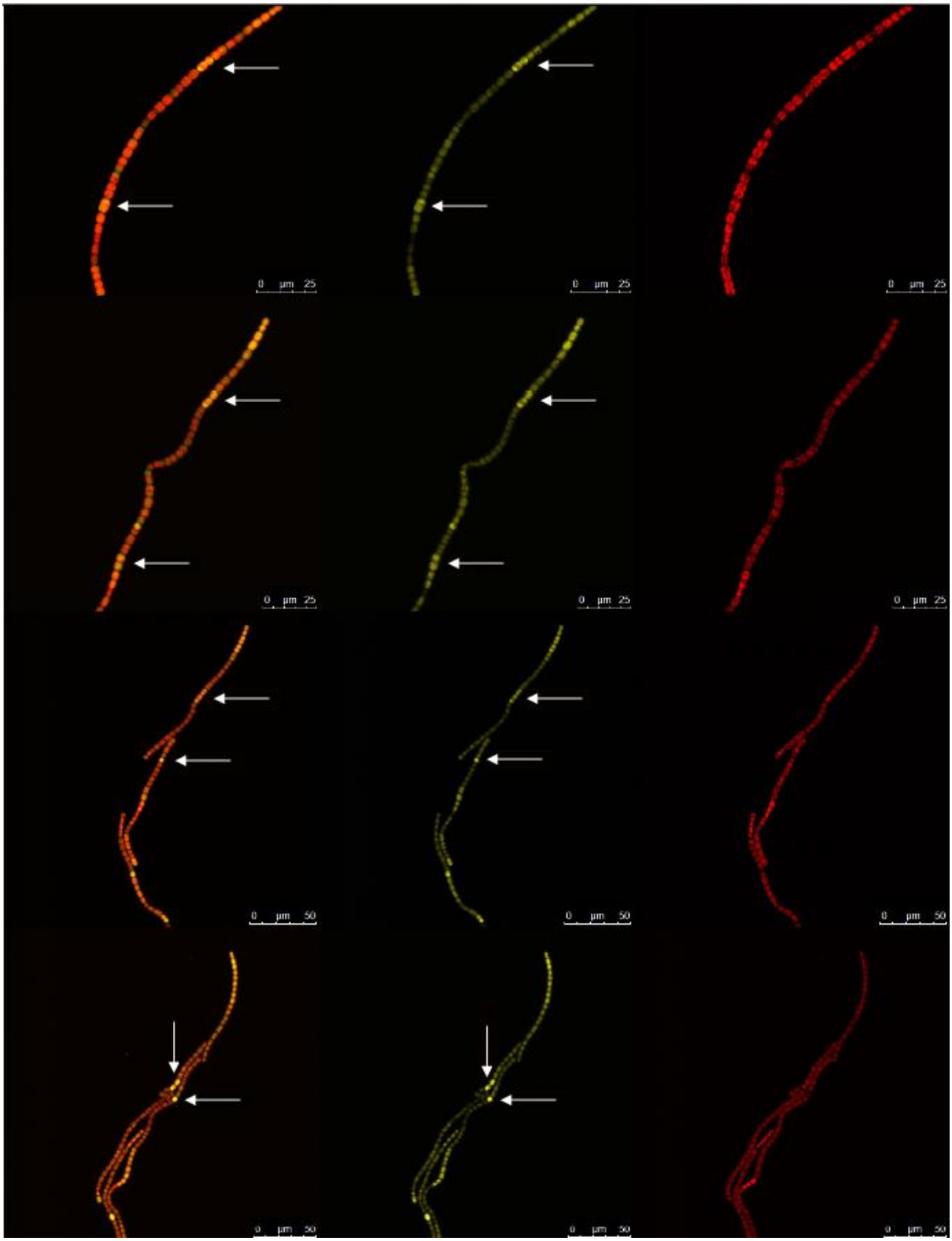


Figure 22: Confocal microscopy pictures depicting the fluorescence of YFP produced using the short native NsiR core promoter of *Anabeana sp*. in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Left to right shows the composite picture, YFP fluorescence and PSII autofluorescence. Top to bottom shows pictures taken at 0, 12, 24 and 48 hours after immobilization. Regions of heterocyst development are here marked with arrows.

2.4.3.4 Synthetic mix promoter

As previously, the expression pattern of the synthetic mix promoter initially showed a number of different cells and regions with enhanced YFP-expression, many of which are close to each other. As time passes and cells split, expression levels in some cells increase while others decrease. Still at twelve hours, many of these cells can still be seen closer to each other than the expected ~10 cells difference (figure 23). As the filament continues to extend, these cells are pulled apart from each other, eventually ending up at the expected distance. As with most of the samples, the filament broke close to a fluorescent cell, most likely because this was a stress point as well as weakening of the cell connection do to the YFP-expression levels.

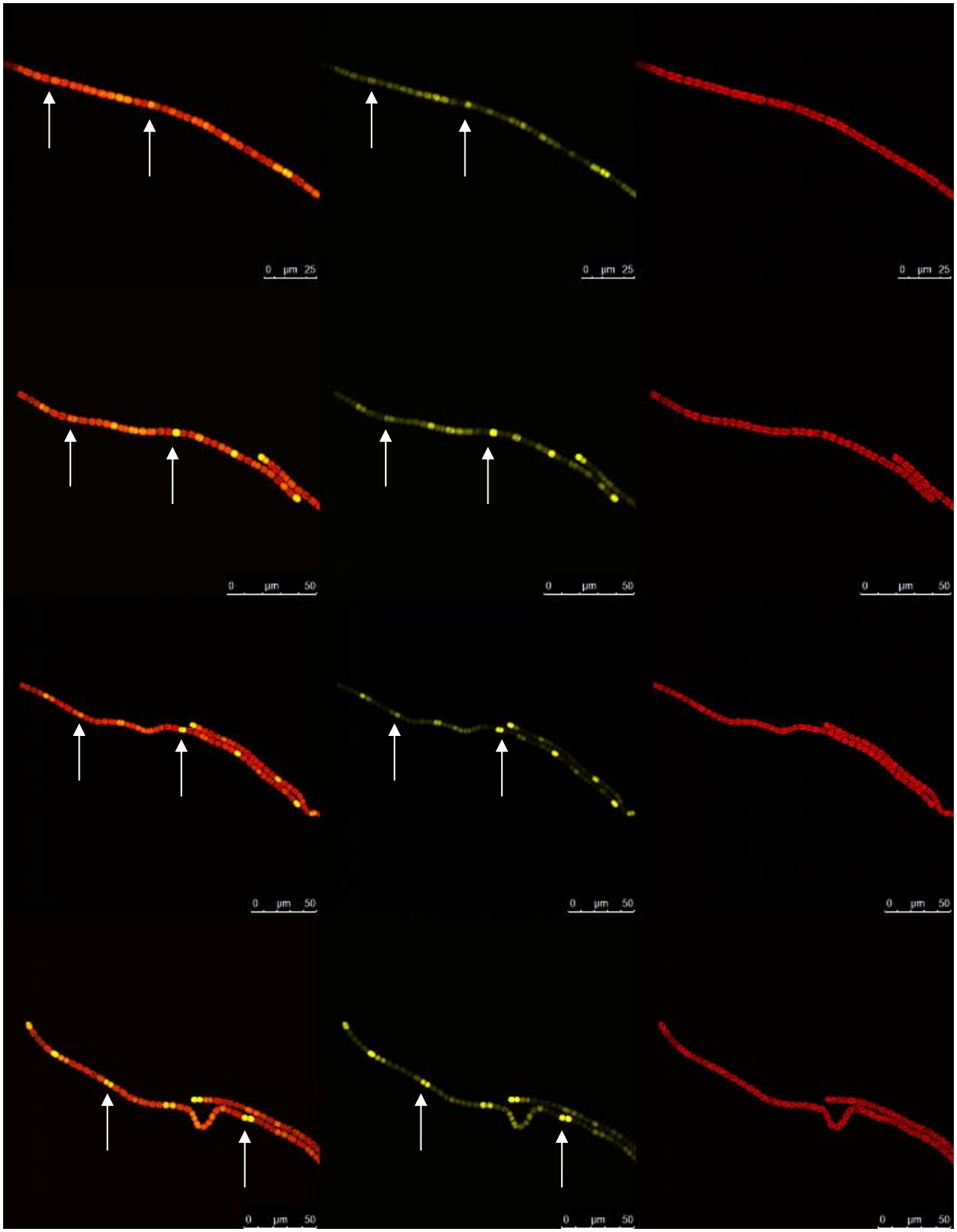


Figure 23: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter featuring a conserved AT-rich UP site in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Left to right shows the composite picture, YFP fluorescence and PSII autofluorescence. Top to bottom shows pictures taken at 0, 12, 24 and 48 hours after immobilization. Regions of heterocyst development are here marked with arrows.

2.4.3.5 -10 change promoter

Once again, the -10 change promoter strain (figure 24) was included as a form of reference to see if there are any behavioral or expression related differences. This construct showed high stability and the filaments did not break at any point during the experiments. At 48 hours it was still impossible to see any indication of heterocyst formation which confirmed the results of the control strain, claiming that too little time had elapsed. We do however not observe the same levels of growth in these cells and many of them appear to be bloated.

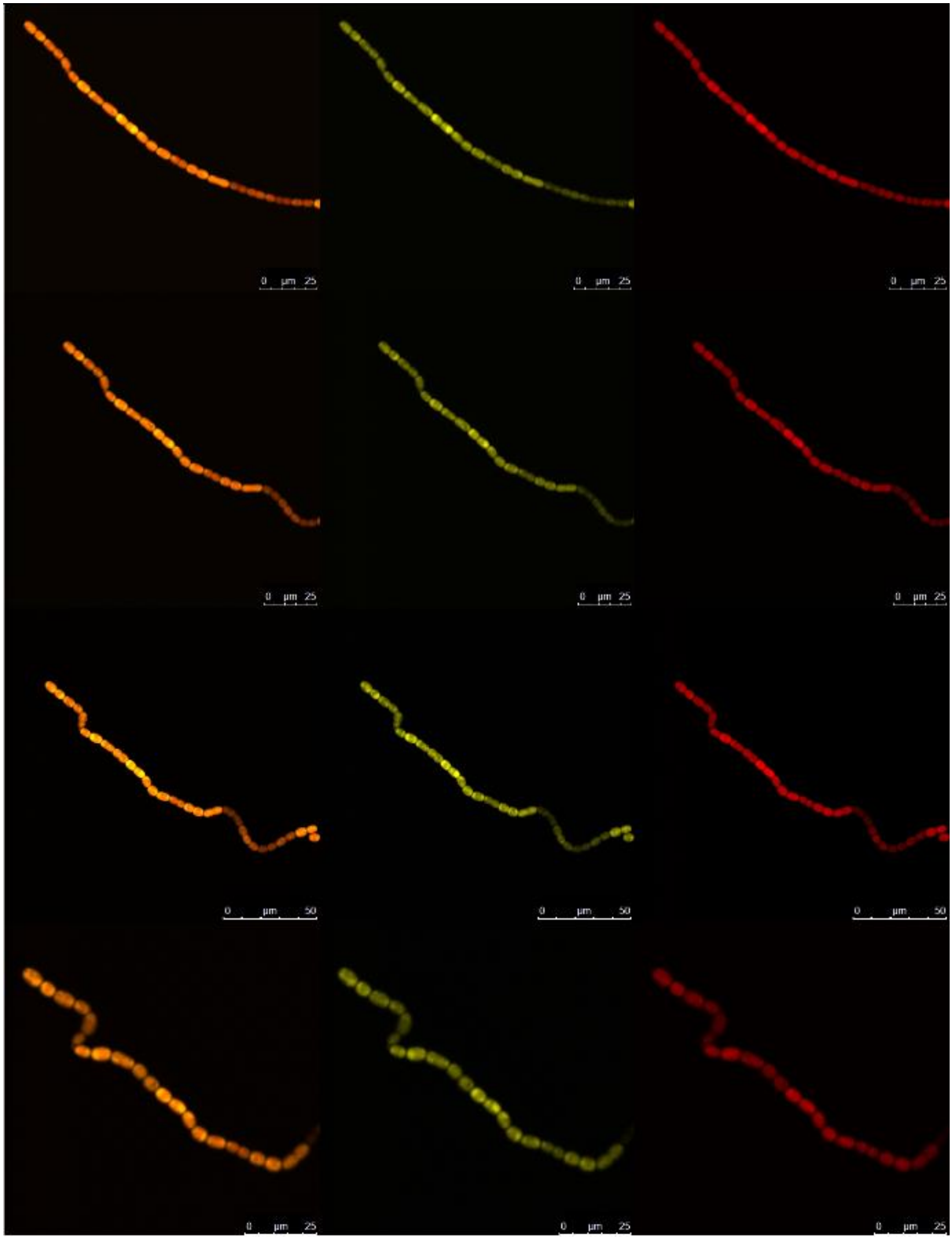


Figure 24: Confocal microscopy pictures depicting the fluorescence of YFP produced using a synthetic promoter featuring the -10 region of P_{trc} in *Nostoc punctiforme*. PSII autofluorescence here shown as red and YFP fluorescence as yellow. Left to right shows the composite picture, YFP fluorescence and PSII autofluorescence. Top to bottom shows pictures taken at 0, 12, 24 and 48 hours after immobilization.

2.5 Quantitative fluorescence measurements

In connection to the confocal microscopy experiments, samples were removed from each strain to enable quantitative measurements of the fluorescence levels at that specific time point. This was done to create an overview in the fluctuations in expression levels throughout heterocyst differentiation. Samples were taken from the initially stepped down cultures and stored in -20 °C until all samples were available for measurement. To ensure comparability between the strains, all samples where normalized with regard to chlorophyll a (chl a) content before introduced into a fluorescence plate reader.

2.5.1 Initial trials

To create a good baseline for the differentiation quantifications, initial experiments were performed on samples grown in ammonium free and ammonium supplied media for 48 hours.

These tests showed a trend towards an increase of fluorescence in nitrogen fixing cells, as compared to ammonium grown, for all samples except for the -10 change (figure 25). This is consistent with results from confocal microscopy which also indicate an increase related to cell specificity. For the -10 change however, we observe the reversed with a dip down to almost half of the original fluorescence when grown under nitrogen fixing conditions (figure 26). This behavior is not possible to observe visually. In addition to this we can observe a remarkable difference in promoter strength. The -10 change promoter produces roughly a tenfold higher fluorescence levels than the other samples with its strong ubiquitous expression. We also observe differences between the three lower yielding constructs where the NsiR I core produced three times that of the mixed synthetic and 6 times that of the fully synthetic strain. Once again this is consistent with the level of specificity and background in the confocal pictures.

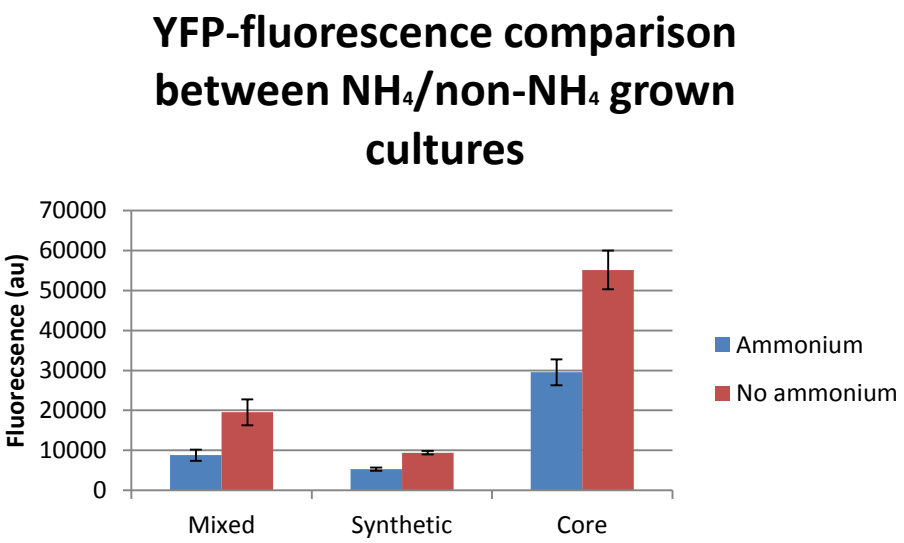


Figure 25: Graph describing the expression levels of YFP measured for experimental promoter strains in *Nostoc punctiforme*. Mixed corresponds to the strain containing the mixed synthetic promoter, synthetic to the fully synthetic promoter and core to the NsiR I core promoter. Results have been compiled from technical triplicates of a single biological sample normalized to a chl a content of 1 mg/ml. Error bars represent one standard deviation.

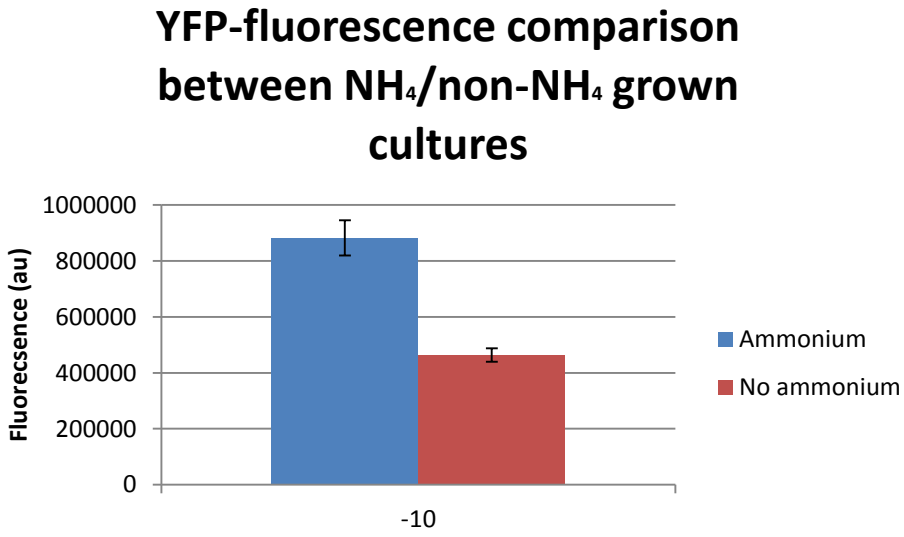


Figure 26: Graph describing the expression levels of YFP measured for experimental promoter strains in *Nostoc punctiforme*. -10 correspond to the strain containing the -10 change promoter. Results have been compiled from technical triplicates of a single biological sample normalized to a chl a content of 1 mg/ml. Error bars represent one standard deviation.

2.5.2 Six hour liquid culture trial

The six hour culture experiments show an increasing trend in the fluorescence of all of the included strains (figures 27-28). A roughly linear behavior is observed for all of the lower yielding strains, while the -10 change strain exhibits a slightly less clear behavior. The increment of the trend line seems to correlate to promoter strength in the sense that an initially higher yielding promoter also exhibits a higher increase over time.

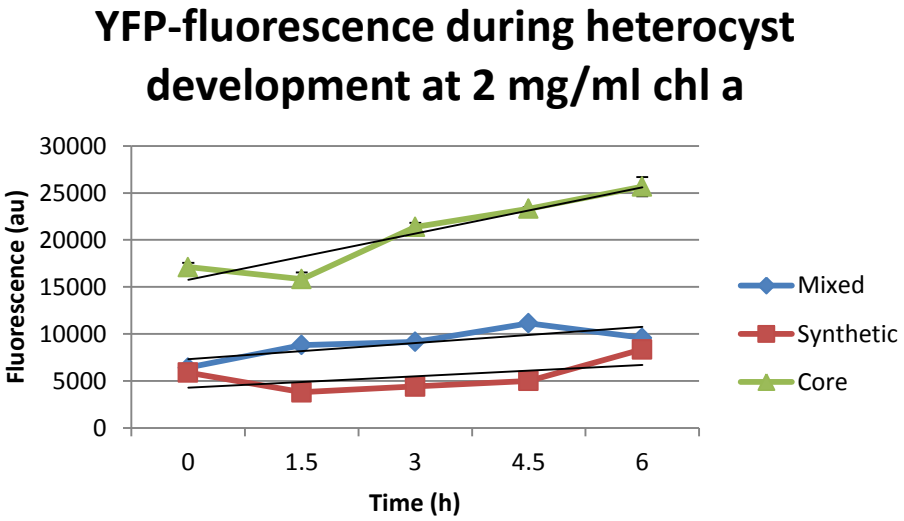


Figure 27: Graph describing the expression levels of YFP measured for experimental promoter strains in *Nostoc punctiforme*. Mixed corresponds to the strain containing the mixed synthetic promoter, Synthetic to the fully synthetic promoter and core to the NsiR I core promoter. Results have been compiled from technical triplicates of biological duplicate samples. Error bars represent one standard deviation. Linear trend lines have been fitted to each sample series.

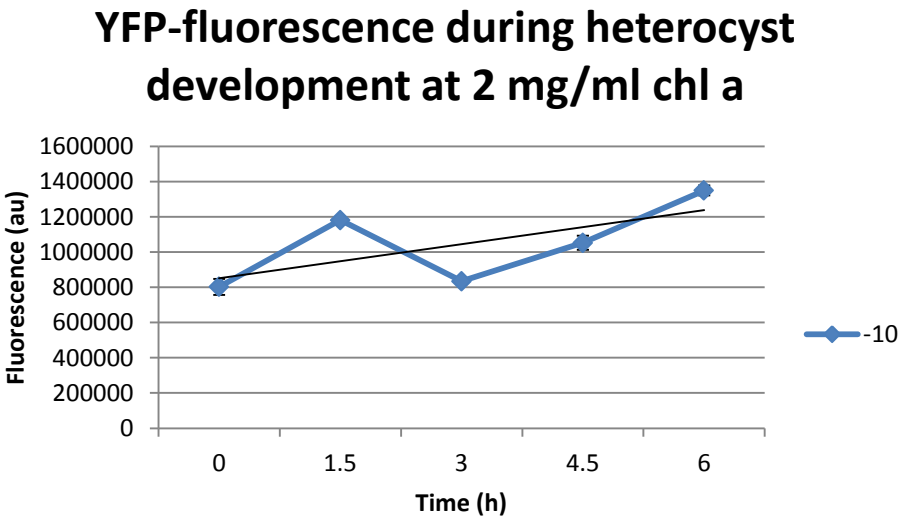


Figure 28: Graph describing the expression levels of YFP measured for experimental promoter strains in *Nostoc punctiforme*. -10 correspond to the strain containing the -10 change promoter. Results have been compiled from technical triplicates of biological duplicate samples. Error bars represent one standard deviation. A linear trend line has been fitted to the sample series.

2.5.3 48 hour immobilized culture experiment

The measurements for the 48 hour experiment were performed after 24 hours and as such the final time point is not present. The lower yielding constructs all showed an increasing trend over this time period with exception of the final time point for the mixed construct (figure 29), when compared to the expected final value from figure 25. It is however possible to determine this point an outlier rather than an actual downwards trend. Observation of the results for the -10 change promoter does on the other hand show a different expression behavior (figure 30). Results indicate parabolic behavior with an increase in the early stages which later once again decreases. Important to note is however that the timescale is shifted for the last two points, meaning that the decrease does not occur at quite as a dramatic rate as might be believed.

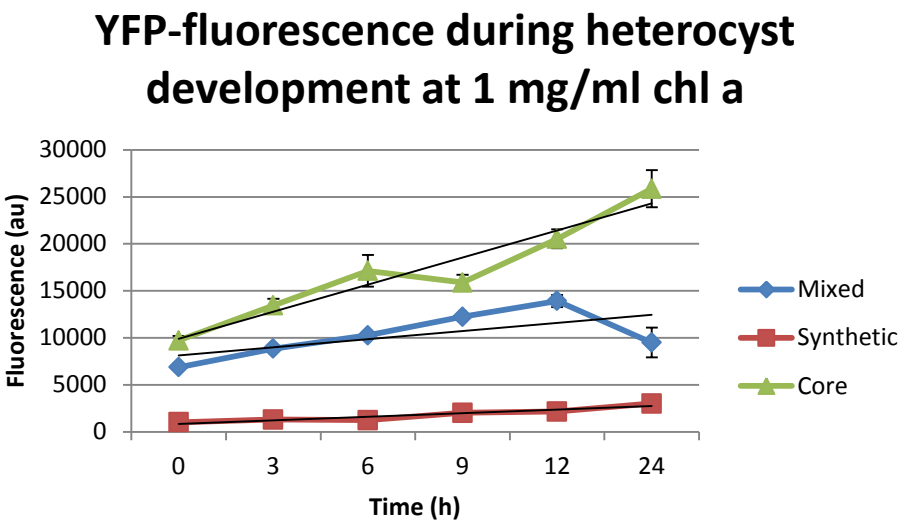


Figure 29: Graph describing the expression levels of YFP measured for experimental promoter strains in *Nostoc punctiforme*. Mixed corresponds to the strain containing the mixed synthetic promoter, Core to the fully synthetic promoter and core to the NsiR I core promoter. Results have been compiled from technical triplicates of a single biological sample. Error bars represent one standard deviation. Linear trend lines have been fitted to the sample series.

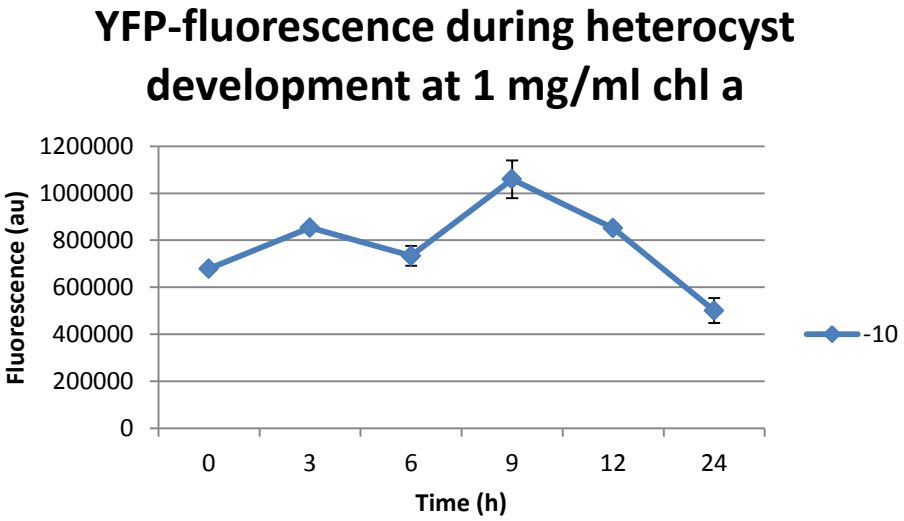


Figure 30: Graph describing the expression levels of YFP measured for experimental promoter strains in *Nostoc punctiforme*. -10 correspond to the strain containing the -10 change promoter. Results have been compiled from technical triplicates of a single biological sample. Error bars represent one standard deviation. A polynomial trend line of the second degree has been fitted to the sample series.

3. Discussion

3.1 Consensus regions provides heterocyst-specific expression

Already in the initial experiments, we proved the function of a fully synthetic heterocyst-specific promoter created using the previously discovered consensus regions (Mitschke *et al.*, 2011)(Appendix A) and spacers consisting of scrambled regions of the NsiR I backbone. This serves as proof that these consensus regions are the only necessary part sequencewise to provide heterocyst-specificity in this particular chassis (figure 7). However using these sequences as opposed to the native versions seems to decrease the expression levels noticeably (figure 24), an effect most likely caused by comparatively lower affinity towards the responsible regulating factors of *N. punctiforme*. This behavior has previously been noted in the binding affinity of sigma factors to the regular bacterial pribnow-box, a sequence that very rarely appears in the fully conserved form (Estrem *et al.*, 1999).

Furthermore, we note that the fully synthetic promoter creates a lot less background fluorescence than the other examined promoters, as well as lacks the observed regional expression enhancement noted in the NsiR I core and mixed synthetic strains (figure 21).

Since the behavior is observable in both the native NsiR I core and the mixed synthetic promoters, which both differ significantly more from each other than they do from the fully synthetic version; it is considered unlikely that the lack of enhanced regions is an effect of the promoter elements themselves. It is deemed more likely that the effect is simply an artifact caused by the low fluorescence levels and that in reality it is present in this sample as well. The observed background fluorescence seems to be caused by some form of leakage in the promoter. Since this is also observed in the NsiR I promoter (figure 13) and the control is completely silent (figure 6), we can conclude that the promoter either has a weak constitutive expression as well or that an important repressor sequence of the original promoter is missing. This effect is most likely also enhanced by the use of a high copy plasmid system. The constitutive part could additionally be activated or repressed by lack or presence of some metabolite causing stochastic expression of the promoter.

It is also observed that the YFP accumulation in the fully synthetic strain can be roughly described in a linear fashion. Observations in figure 30 additionally indicate that YFP seems to start to breakdown after roughly 9 hours *in vivo*. This would point towards an increase in the expression of the fluorophore in the filaments over time (figure 29). Confocal microscopy pictures hint that this might be explained by cell exclusive expression (figure 21). Assuming that the background fluorescence is at equilibrium before the first measurement, this would indicate a clear “on” signal for expression in certain cells which then gradually increases during differentiation.

Finally, an observation of a strongly fluorescent cell of the synthetic strain dividing was made at roughly 12-24 hours (figure 17) indicating that growth is still present even at this stage. This in turn proves that the cells are yet to morphologically become pre-heterocyst, which do not possess this ability. It also gives a possible explanation to why pre-heterocysts are sometimes found next to each other prior to full maturation.

3.2 Addition of an UP-element increases expression

Similar to the fully synthetic strain, the mixed synthetic proved strong YFP over expression in specific cells already from the beginning, but features a shorter distance between these cells than what was to be expected. A lot clearer background fluorescence and regional enhancement of the expression in stretches of 3-5 cells can be observed in the early stages (figures 24), as well as a roughly twofold increase in final fluorescence levels compared to the fully synthetic version (figure 25) The increase in quantitative expression levels can be explained by the introduction of the AT-rich up element, serving to enhance the binding of RNA-polymerase and thus increasing the protein yield (Huang and Lindblad, 2013). If we combine this observation with the conclusion that the DIF motif and Pribnow-box are solely responsible for heterocyst-specificity in this system, our result points towards the system being regulated by a sigma factor.

Observation over 48 hours indicates that strongly fluorescent cells of the mixed synthetic strain do not revert in fluorescence but rather stay in this state while surrounding vegetative cells divide and provided proper spacing. This would indicate that once a cell has received induction it proceeds to morph at least into a pre-heterocyst. The enhanced regions on the other hand seem to either revert or develop into one or two strongly fluorescent cells (figure 25). In this particular sample it was also observed how a previously non-fluorescent terminal cell quickly gained fluorescence as soon as there was proper spacing between it and the closest fluorescent cell (figure 19). This might be related to the fact that the important factors for inhibition of heterocyst differentiation tend to diffuse outwards from pre-heterocysts. In the case of a terminal cell, it can only receive input from one end and as such inherently receives less (Risser and Callahan, 2009).

These results all strengthen the hypothesis stated regarding the synthetic promoter, that there is some form of “on” signal expressed in certain cells, which in the case of mixed synthetic strain seems to behave a lot more stochastically in where it is expressed in early stages of nitrogen starvation. This might somehow be related to the introduction of the UP element, which theoretically should also increase the likelihood of transcription of the gene. Regions where no strongly fluorescent cells are present then develop heterocysts in a much more controlled way as with the terminal one in figure 19.

Quantitative fluorescence experiments in figure 29 indicate a dip in fluorescence towards 24 hours. Comparing to the initial experiments in figure 25, we can however conclude that this is likely to be an outlier, since the overall expression is still expected to be increasing at this point.

3.3 The pribnow-box affects expression pattern

The -10 change promoter displays very different behavior when compared to the other constructs. The introduction of the pribnow-box of a strong constitutive promoter totally removes specificity as well as multiplies the expression levels by a factor of roughly 100 (figures 10 and 26.). This shows us both that DIF-containing promoters can be expressed outside of heterocysts, as well as indicates that the DIF motive alone is not solely responsible for heterocyst-specific expression in this particular system. Additionally, the strain is very useful as a positive reference when trying to determine what is a relevant shift in background fluorescence and what is simply a natural variation either caused by the positioning of the filament or light scattering in the microscope.

Another interesting observation is found when studying the quantitative expression levels of the -10 change strain. In figure 26 we find that the expression levels decrease when the cells are grown under nitrogen fixing conditions, this indicates that the promoter might somehow be regulated by the access to nitrogen. Since no known connection between the P_{trc10} promoter and nitrogen has previously been reported, we can assume that the DIF region is in some form involved in this regulation either directly or indirectly. This would indicate that the native promoter features both heterocyst-specific features as well as regulation through the presence of nitrogen in the surrounding. It has previously been described by Mitschke *et al.* (2011) that the DIF motif sometimes overlaps with the binding site of the nitrogen-responsive regulatory protein, NtcA which might be a possible cause of this effect.

Observing the expression behavior in the -10 change strain over 24 hours (figure 30) indicate that we have an accumulation of YFP when depleted of nitrogen which then follows a parabolic downwards trend. This means that the expression of YFP is activated at nitrogen starvation and then slowly declines with time. This compared with results from the other promoters (figure 29) indicates that nitrogen plays a key part in the initial activation of the promoter and that some other factor later takes over. This in turn might explain the stochastic expression pattern of the mixed synthetic and NsiR I promoters.

The lack of growth and bloating of the cells observed in the immobilization experiment of the -10 strain is most likely to be caused by the strain of expressing high levels of YFP. Increased internal pressure could have caused the cells to swell.

3.4 General observations

Some general observations regarding the behavior of the filaments were noticed throughout the experiments. It has previously been described by (Muro-Pastor, 2014) that expression of the native promoter as well as the first signs of heterocyst differentiation in liquid culture should be noticeable after roughly 6 hours. In these experiments we do however find noticeable differences in expression behavior much earlier than so, most of the time even directly after initiation (figure 13). Additionally, it is very hard to determine any decrease in the auto-fluorescence of the cells at this time. The early noticeable expression pattern can be caused by the use of the high-copy plasmid system where low level expression is biologically multiplied. This theory is strengthened by the difference in observed background fluorescence in the different strains. The lack of receding auto-fluorescence on the other hand might be caused by the fluorescence spillover observed in figure 11, where strong YFP fluorescence may create observed expression in the auto-fluorescent spectrum. It is possible that this effect occurs even at low level fluorescence even though it does not become prominent until much later. What causes this spillover is hard to pinpoint since spectra of the two fluorescence reading filters in the confocal microscope do not overlap (see material and methods 4.17). Most likely, the error lies somewhere in the function or handling of the instrument.

Another important observation lies in the behavior of the filaments during different treatment in the microscope. During the initial experiments it was noticed that growth in small six-well plates caused the cells to clump together and become difficult to analyze. This behavior is common when stirring is low and is simply an effect of knotting and sticky surface polysaccharides (Otero and Vincenzini, 2003). Additionally we saw that filaments tended to move considerably during the process, making it near impossible to get good contrast in the pictures. Since no fixation of cells was performed, this is to be expected due to the residual liquid. If following experiments were to only be performed once, it is possible that fixation of the sample could have been performed by letting the slides dry prior to microscopy. This is however time consuming and removes the ability to observe the same sample several times. Additionally it disrupts the natural growth environment.

Later trials with the immobilization on agar plates solved these issues as well as introduced the possibility of filament-specific observations. However it was noticed that cultures grown in this fashion causes the filaments bend in a zigzag and eventually break. This is thought to be due to the fact that cells find it harder to penetrate forward into the gel rather than simply moving towards the sides (figure 16). When compared to growth in liquid culture it also seems that differentiation occurs at a much slower rate. Since the strain is native to an aquatic environment, this is to be expected.

3.5 Future studies

To be able to make proper conclusions regarding the function of each individual promoter element, more combinations of these need to be tested.

First of all, confocal microscopy and quantitative measurements on the long native NsiR I promoter should be performed to be able to determine how its expression compares to the core version as well as the mixed synthetic. Such investigations could provide confirmation regarding all conclusions drawn from previously examined samples since it provides a missing link between them. Current setup lacks a control for the function of the UP-element.

Furthermore a weaker non-heterocyst-specific -10 region should be tested to determine if the constitutive behavior persists or if it was simply an effect of the P_{trc} pribnow-box overpowering the system. In combination with this it is also suggested to examine the effect on heterocyst-specificity of switching out the DIF-motif for another -35 region although it has already been proved by Li *et al.* (2015) that fully removing the motif removes specificity. It would also be interesting for future application to test integration of other motifs into the system, such as regulating motifs.

In light of the results of the confocal microscopy where it was discovered that differentiation was not complete after 48 hours on plates, it can be concluded that there is a need to further prolong the experimental time. However after 48 hours, substantial breakage is observed in the filaments as well as that the plates have started to dry out. This in combination with the inherent difficulties to uphold the same level of confidence of the experimentalist over a long experiment makes simply adding a day to the experiment suboptimal. As such, a possible method to solve this is to simply let the cultures grow in liquid media for a few hours before immobilization. This would forward the development somewhat but not too far as indicated by the six hour experiments and enable complete differentiation experiments in the same timeframe as before.

Quantitative measurements with shorter intervals between them could also be of use to determine the true behavior of the system. This is especially interesting for the -10 change strain since it shows such a different behavior.

To be able to truly understand the regulation of the promoter, it is of the utmost importance to identify the regulating transcription factors. The conserved motifs indicate that the promoter is regulated via a separate sigma factor but there is no conclusive evidence for this as of yet. This project initially incorporated the identification of this factor by a protein-DNA affinity assay, but due to time constraints it was not performed. The method included immobilization of the promoter on magnetic beads through biotin-streptavidin interaction. The beads would then be flushed with protein extract and the regulating factors would theoretically stick to the promoter segment. This could later be fished out using a magnet, removed from the beads and analyzed through mass spectrometry and electromobility shift assays. This still needs to be performed to provide true insights to the actual functions.

3.6 Conclusions

This report has served to investigate and determine the effects of specific motifs of the heterocyst-specific NsiR I promoter of *Anabaena sp. 7120.* This was performed with the purpose of increasing knowledge regarding what, sequencewise, creates a heterocyst-specific expression as well as investigating whether it was possible to create a fully synthetic promoter that retains specificity through the use of consensus versions of conserved promoter regions. Investigation into this subject is of interest for the potential use of heterocysts in filamentous cyanobacteria as anaerobic production compartments for oxygen sensitive compounds and/or machinery.

It is here shown that incorporation of the most highly conserved promoter regions into a scrambled NsiR I chassis, creates a functional promoter with high cell specificity. This in turn proves that these two regions which are located at -10 and -35 bp upstream of the transcription start site are the only necessary components to create heterocyst-specificity in this system. Additionally, it is shown that the -35 region alone is not enough to retain this behavior when the -10 region is switched to that of a strong constitutive promoter, indicating that these two might somehow interact. When combined with the discovery that expression levels of the synthetic promoter is doubled when an AT rich upstream region is introduced, it seems likely that regulation of this promoter, and subsequently heterocyst-specificity in general, is driven by a sigma factor.

Investigations of the quantitative expression patterns showed that a promoter variant containing the conserved -35 region and the -10 region of a strong constitutive promoter displayed a parabolic behavior when grown under nitrogen fixing conditions. This along with the observation of a linear expression pattern in the fully synthetic promoter gives an indication that expression might be activated by deprivation of nitrogen. In the case of the promoter with an introduced constitutive region, expression following induction then decreases with time. Combined with the knowledge that this promoter has a constitutive expression pattern we can deduce that the native promoter might be regulated in a tandem fashion which is originally driven by the loss of nitrogen and later by pre-heterocyst-specific compounds.

It was also observed that certain stochasticity is present in regard to which cells originally start to express fluorescence on this system. It is possible for cells that are originally situated closer than the expected distance between heterocysts to show expression. Rather than having one of them revert back into a non-fluorescing cell, it seems that these cells continue to express fluorescence while the vegetative cells between them split, eventually creating the proper distance. In addition it was observed that terminal cells in a filament features a higher frequency in development into pre-heterocysts and that splitting of highly fluorescent cells is still possible in the early stages causing a paired pre-heterocyst phenomenon.

Finally, during this project a new practical method to observe single stationary filaments over a time period of 48 hours, was developed. This method allows pinpoint accuracy and can be utilized for single cell microscopy as well as observation of larger structures.

Even though this report provides rather extensive documentation, more experiments are still required to provide substantial evidence for the observed results. Essential in future experiments is the identification of the transcriptional regulator itself which in turn can be used to test the concluded observations in this report.

4. Materials and Method

4.1 Instruments

Spectrophotometer

Varian cary 50 bio UV-visible spectrometer

Thermo scientific nano-drop 2000 UV-visible spectrometer

Thermocycler

Biorad MJ mini gradient thermal cycler

Sonicator

Sonics Vibra-cell VC 130

Electroporator

Bio Rad Gene Pulser Xcell Electroporation System

Confocal microscope

Leica DM6000 CS confocal microscope

Plate reader

Hidex Chameleon 4.47

4.2 Web tools used for sequence alignment and study of sequence conservation

MUSCLE (MUltiple Sequence Comparison by Log- Expectation) (Edgar, 2004)

Weblogo (Crooks *et al.*, 2004)

4.3 Bacterial strains

Escherichia coli DH5α (Invitrogen, 2013)

Nostoc punctiforme wild type (Rippka *et al.*, 1979)

Strain description	**Strain Name**
N. punctiforme containing a promoterless PAW_YFP plasmid with intact ccdB operon.	MIS_Npun_YFP_Neg
N. punctiforme containing a PAW_YFP plasmid where the YFP expression is driven by the NsiR I (core) promoter.	MIS_Npun_YFP_Core
N. punctiforme containing a PAW_YFP plasmid where the YFP expression is driven by the NsiR I (long) promoter.	MIS_Npun_YFP_Long
N. punctiforme containing a PAW_YFP plasmid where the YFP expression is driven by a synthetic promoter created using the DIF motif, a pribnow-box that is conserved in heterocyst-specific promoters and the skeleton of NsiR I (core)	MIS_Npun_YFP_Syn
N. punctiforme containing a PAW_YFP plasmid where the YFP expression is driven by a synthetic promoter created using the DIF motif, a pribnow-box and an UP element that are conserved in heterocyst-specific promoters and the skeleton of NsiR I (core)	MIS_Npun_YFP_Mix
N. punctiforme containing a PAW_YFP plasmid where the YFP expression is driven by a synthetic promoter created using the DIF motif, the pribnow-box of P_{trc} and the skeleton of NsiR I (core)	MIS_Npun_YFP_-10

4.4 Cyanobacterial cultivation

On plates

The cultivation of cyanobacterial cultures on plates was performed at 30°C on $BG11_0$(Appendix B) agar medium supplemented with 5 mM NH_4Cl and 10mM HEPES. When required for selection purposes, 25-50 μg/ml neomycin was added to the mixture.

In liquid

The cultivation of cyanobacterial cultures in liquid was performed on shakers (120 rpm) at 30°C either in sterile 125 ml e-flasks or in 10 ml six-well plates.

In the case of e flask, cultivation took place in 50 ml $BG11_0$ supplemented when appropriate with 5 mM NH_4Cl, 10mM HEPES and 25-50 μg/ml neomycin.

In the case of six-well plate, cultivation took place in 6 ml $BG11_0$ supplemented when appropriate with 5 mM NH_4Cl and 10mM HEPES and 25-50 μg/ml neomycin.

4.5 *Escherichia coli* cultivation

The cultivation of *E. coli* cultures on plates was performed at 37°C on LB agar medium supplemented with 50 mg/ml kanamycin for selection purposes.

4.6 Restriction digest

Restriction digest was performed according to manufacturer instructions with the use of Thermo Scientific Fast Digest enzymes.

4.7 Purification of restriction digests

Purification of restriction digest was performed according to manufacturer instructions with use of Thermo Scientific GeneJET PCR purification kit. Deionized water was used for elution to avoid salts in the sample.

4.8 Ligation

Ligation was performed with the use of New England Biolabs QuickLigase, according to manufacturer instructions.

4.9 Transformation of *Escherichia coli*

E. coli transformation was performed following the suggested protocol "4.12 *E. coli* transformation" as described by Ievgen Dzhygyr, (2013) in his master's degree project.

4.10 Colony PCR

Colony PCR was performed following the suggested protocol "4.13 Colony PCR (*E.coli* and cyanobacteria)" as described by Ievgen Dzhygyr, (2013) in his master's degree project.

4.11 Plasmid extraction

DH5α *E. coli* was utilized as host for plasmid cloning. 20 ml of LB supplied with 50 mg/ml kanamycin was inoculated in a 50 ml falcon tube by scraping bacteria from a plate with a pipette tip. Cultivation was performed overnight and cells were harvested through centrifugation at 3500 g. Extraction of the plasmid was then performed using the GenJET Plasmid Miniprep Kit from Thermo Scientific according to manufacturer instructions. Elution was performed with deionized water and plasmids were stored at -20°C.

4.12 Sequencing

All sequencing was performed by Macrogen Europe Inc.

4.13 Transformation through electroporation

The desired plasmids were purified at high concentration (>1µg/ml). 50 ml of thick *N. punctiforme culture* was spun down at low g or left to sediment for 20 minutes before the supernatant was removed. The remaining cell pellet was cooled on ice and then sonicated (Amp 20-30, pulse 1p 1.0 sec) 3 times for 30 sec. Following sonication, the pellet was resuspended in 25 ml ammonium supplied $BG11_0$ and left to recover for 4 hours or overnight at 30°C in weak illumination. The culture was washed with new media through centrifugation 4 times, before finally being pelleted. Roughly 100 µl were then transferred to precooled eppendorf tubes which were then supplied with 2 µg of desired plasmid. The mixture was then transferred to a pre-cooled electroporation-cuvette and electroporated with the following settings: 2400 V, Ts 5.0, 2mm. Electroporated cells were then transferred to a sterile e-flask and left to recover overnight under previously described conditions. Finally, the cells were once again spun down and the resulting pellet was spread on cellulose filters on $BG11_0$ plates supplied with ammonium and neomycin. The plates were left in weak illumination until single cell colonies could be observed.

4.14 Chlorophyll a measurements

1 ml of cyanobacterial culture was placed in a 1.5 ml centrifuge tube. The cells were then pelleted for 2 minutes at maximum rpm in a centrifuge before 900 µl of the supernatant was removed. The removed liquid was replaced by 900 µl of 100% methanol and vortexed vigorously followed by 15 minutes of incubation in darkness. The mixture was then once again spun down for two minutes and 900 µl of supernatant was removed and placed in a plastic cuvette. Chl a has a characteristic absorbance peak at 665 nm (figure 2) and this was used to quantify the sample concentration through the use of equation 1.

$$mg/ml\ chl\ A = 12.7 * abs665$$

Equation 1: Equation describing a simple and crude way to quantify chlorophyll a content in a sample. The constant relates to the extinction coefficient of chlorophyll a (1/78.74 x 103) as described by Meeks and Castenholz (1971).

4.15 Quantitative fluorescence measurements

For the quantitative fluorescence measurements, 1.5 ml of liquid was removed from the cyanobacterial culture and homogenized thoroughly with a syringe. 1 ml of the removed sample was used for measuring the levels of chl a as described in 4.14 while the other 0.5 ml was mixed with water to a final concentration of 1 mg/ml chl a. Triplicates of 200 µl each were transferred into a 96-well plated which was then analyzed in a plate reader with an excitation filter at 485 nm and an emission filter at 535 nm. To ensure proper mixture, the machine was set to shake the plate between each separate measurement point.

4.16 Preparation of samples for microscopy

Samples were prepared in three different ways depending on experiment.

In the initial experiment, 1 ml of cyanobacterial culture was removed from the sample e-flask and left to sediment. 20 µl of the culture sediment was then transferred onto a glass slide and covered with a cover glass which was introduced to the microscope.

For the 6 hour trial, the strains were grown similarly in e-flasks until desired cell density and then nitrogen stepdowned through 4 centrifugations with removal of present supernatant and addition of fresh ammonium free media between each. 1 ml of culture was then similarly removed and left to sediment. A rectangular piece of agar gel was cut with a scalpel from a precast plate and transferred to a glass slide. 20 µl of the culture sediment was then transferred on top of the gel which was then covered by a cover glass. The cover glass was given a gentle squeeze to remove excess liquid from the specimen and was then introduced to the microscope.

For the final 48 hour experiment, the strains were as before pre grown in e-flasks and stepped down to remove excess ammonium. While this was done, small $BG11_0$-agar gel plates were cast in the lids of mini petri-dishes. The lids were filled to the brim to create a flat surface similar to that of a glass slide so that the edges of the plate would not interfere with the machinery of the microscope. 200 µl of sample was then carefully spread on each plate and a promising region was covered with a cover glass. The plates were left to dry briefly to fully immobilize the cells and remove excess liquid before being introduced to the microscope. When a single free filament had been found, the edges of the plates were marked to ensure the same positioning in the microscope every time and coordinates for the filament to be followed was logged. As such it was possible to return to the exact same filament for each image. Between pictures, the plates were kept in a sealed plastic box with a water supply to ensure that they did not dry out. The main drawback of this method is that the thick agar plate does not allow the light from the bottom illuminator of the microscope to pass through properly, thus removing the possibility to use a brightfield filter.

4.17 Confocal microscopy

All confocal microscopy was performed utilizing excitation with a green argon laser at 514 nm and a pinhole setting of Airy 1. Emission for YFP and autofluorescence was collected at the wavelengths described in Figure 31. Individual adjustments were performed to improve contrast in each sample by manipulating fluorescence intensity *in silico* and as such no quantitative conclusions can be drawn from the pictures. Pictures were taken with a pixel size of 512x512 at a sampling rate of 400 Hz.

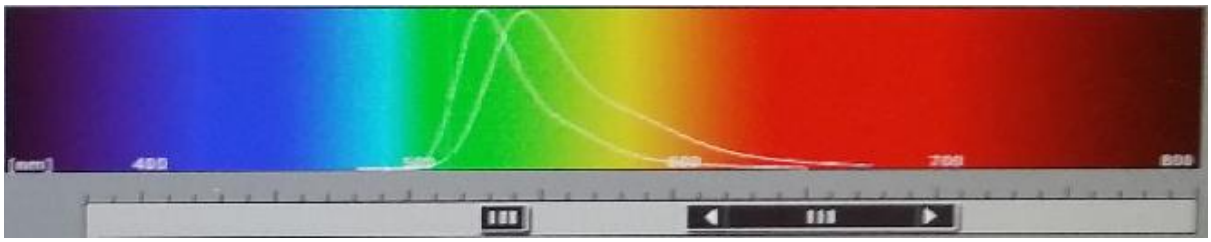


Figure 31: Description of the emission spectra of GFP and YFP with associated collection areas of the fluorescence detectors. The left peak describes the emission of GFP and the right that of YFP. The sliding bars below represent the wavelengths collected by the individual detector. The left shows the collection area of the YFP detector with ranging from its theoretical emission peak at 527 nm to roughly 540 nm. The right shows the collection area of the autofluorescence detector and spans between 600 and 700 nm.

References

Browning, D.F., and Busby, S.J.W. (2004). The regulation of bacterial transcription initiation. Nature Rev. Microbiol. 2, 57-65.

Carlsson, K., Danielsson, P.E., Liljeborg, A., Majlöf, L., Lenz, R., and Åslund, N. (1985). Three-dimensional microscopy using a confocal laser scanning microscope. Opt. Lett. *10*, 53.

Copertino, D.W., Edelman, G.M., and Jones, F.S. (1997). Multiple promoter elements differentially regulate the expression of the mouse tenascin gene. Proc. Natl. Acad. Sci. U. S. A. *94*, 1846–1851.

Crooks, G.E., Hon, G., Chandonia, J.-M., and Brenner, S.E. (2004). WebLogo: a sequence logo generator. Genome Res. 14, 1188–1190.

Dzhygyr I. (2013). DPS Proteins Impact on Oxidative Stress Response in Nitrogen Fixing Cyanobacterium Nostoc punctiforme ATCC 29133, (p11-13).

Edgar, R.C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res. 32, 1792–1797.

Estrem, S.T., Ross, W., Gaal, T., Chen, Z.W.S., Niu, W., Ebright, R.H., and Gourse, R.L. (1999). Bacterial promoter architecture: subsite structure of UP elements and interactions with the carboxy-terminal domain of the RNA polymerase alpha subunit. Genes Dev. *13*, 2134–2147.

Flanagan, K.A., Comber, J.D., Mearls, E., Fenton, C., Erickson, A.F.W., and Camp, A.H. (2016). A Membrane-Embedded Amino Acid Couples the SpoIIQ Channel Protein to Anti-Sigma Factor Transcriptional Repression during Bacillus subtilis Sporulation. J. Bacteriol. *198*, 1451–1463.

Gruber, T.M., and Gross, C.A. (2003). Multiple Sigma Subunits and the Partitioning of Bacterial Transcription Space. Annu. Rev. Microbiol. *57*, 441–466.

Heidorn, T., Camsund, D., Huang, H.-H., Lindberg, P., Oliveira, P., Stensjö, K., and Lindblad, P. (2011). Synthetic Biology in Cyanobacteria: Engineering and Analyzing Novel Functions. In Methods in Enzymology, Vol 497: Synthetic Biology, Methods for Part/Device Characterization and Chassis Engineering, Pt A, C. Voigt, ed. (San Diego: Elsevier Academic Press Inc), pp. 539–579.

Huang, H.-H., Camsund, D., Lindblad, P., Heidorn, T. (2010). Design and characterization of molecular tools for a Synthetic Biology approach towards developing cyanobacterial biotechnology. Nucleic Acids Res 38, 2577 –2593.

Huang, H.-H., and Lindblad, P. (2013). Wide-dynamic-range promoters engineered for cyanobacteria. J. Biol. Eng. 7, 10.

Invitrogen (2013) DH5α Competent Cells. [Web document]
URL:http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/competent-cells- for-transformation/competent- cells-strains/dh5a-competent-cells.html. Date visited 16.06.2016.

Ionescu, D., Voss, B., Oren, A., Hess, W.R., Muro-Pastor A.M. (2010). Heterocyst-Specific Transcription of NsiR1, Non-Coding RNA Encoded in a Tandem Array of Direct Repeats in Cyanobacteria. J. Mol. Biol. 398 (2): 177-188

Kennell, D., and Riezman, H. (1977). Transcription and translation initiation frequencies of the Escherichia coli lac operon. J. Mol. Biol. 114 (1): 1–21.

Lakowicz, J.R. (2013). Principles of Fluorescence Spectroscopy (Springer Science & Business Media).

Li, X., Sandh, G., Nenninger, A., Muro-Pastor, A.M., and Stensjö, K. (2015). Differential transcriptional regulation of orthologous dps genes from two closely related heterocyst-forming cyanobacteria. FEMS Microbiol. Lett. 362.

Meeks, J.C., Castenholz, R.W. (1971). Growth and photosynthesis in an extreme thermophile, Synechococcus lividus (Cyanophyta). Arch Mikrobiol. 78(1), 25-41.

Meeks, J.C., Elhai, J., Thiel, T., Potts, M., Larimer, F., Lamerdin, J., Predki, P., and Atlas, R. (2001). An overview of the genome of *Nostoc punctiforme*, a multicellular, symbiotic cyanobacterium. Photosynth. Res. *70*, 85–106.

Mitschke, J., Georg, J., Scholz, I., Sharma, C.M., Dienst, D., Bantscheff, J., Voss, B., Steglich, C., Wilde, A., Vogel, J., *et al.* (2011). An experimentally anchored map of transcriptional start sites in the model cyanobacterium Synechocystis sp PCC6803. Proc. Natl. Acad. Sci. U. S. A. *108*, 2124–2129.

Muro-Pastor, A.M. (2014). The Heterocyst-Specific NsiR1 Small RNA Is an Early Marker of Cell Differentiation in Cyanobacterial Filaments. Mbio *5*, e01079–14.

Otero, A., and Vincenzini, M. (2003). Extracellular polysaccharide synthesis by Nostoc strains as affected by N source and light intensity. J. Biotechnol. *102*, 143–152.

Pribnow, D. (1975). Nucleotide sequence of an RNA polymerase binding site at an early T7 promoter. Proc. Natl. Acad. Sci. U. S. A. *72*, 784–788.

Rippka, R., Deruelles, J., Waterbury, J., Herdman, M., and Stanier, R. (1979). Generic Assignments, Strain Histories and Properties of Pure Cultures of Cyanobacteria. J. Gen. Microbiol. *111*, 1–61.

Risser, D.D., and Callahan, S.M. (2009). Genetic and cytological evidence that heterocyst patterning is regulated by inhibitor gradients that promote activator decay. Proc. Natl. Acad. Sci. U. S. A. *106*, 19884–19888.

Scholten, M., and Tommassen, J. (1994). Effect of Mutations in the -10-Region of the Phoe Promoter in Escherichia-Coli on Regulation of Gene-Expression. Mol. Gen. Genet. *245*, 218–223.

Shaner, N.C., Steinbach, P.A., and Tsien, R.Y. (2005). A guide to choosing fluorescent proteins. Nat. Methods *2*, 905–909.

Sheen, J., Hwang, S., Niwa, Y., Kobayashi, H., and Galbraith, D. (1995). Green-Fluorescent Protein as a New Vital Marker in Plant-Cells. Plant J. *8*, 777–784.

Tamagnini, P., Axelsson, R., Lindberg, P., Oxelfelt, F., Wunschiers, R., and Lindblad, P. (2002). Hydrogenases and hydrogen metabolism of cyanobacteria. Microbiol. Mol. Biol. Rev. *66*, 1 – +.

Thiel, T. (2004). Nitrogen Fixation in Heterocyst-Forming Cyanobacteria. In Genetics and Regulation of Nitrogen Fixation in Free-Living Bacteria, W. Klipp, B. Masepohl, J.R. Gallon, and W.E. Newton, eds. (Springer Netherlands), pp. 73–110.

Tsien, R.Y. (1998). The green fluorescent protein. Annu. Rev. Biochem. *67*, 509–544.

Yakovchuk, P., Protozanova, E., and Frank-Kamenetskii, M.D. (2006). Base-stacking and base-pairing contributions into thermal stability of the DNA double helix. Nucleic Acids Res. *34*, 564–574.

Acknowledgements

First of all I would like to thank my supervisor Karin Stensjö for the chance to work in her group with this project. It has been immensely valuable for me to be able to discuss ideas and results with you throughout my time in the lab. I am very grateful for the moral support you provided when results were not in my favor and for the freedom I was given to test my own ideas. I believe that you have helped me develop myself both as a person and as a scientist.

I would like to thank Xin Li for the close collaboration we have had during this project. You have provided a lot of practical knowhow around the lab and solved my experimental problems when I was at a loss. Furthermore, you have helped keeping my mood up when I was frustrated with the slow progress of my experiments.

I would like to thank Adam Wegelius for providing the plasmid used in the project as well as for providing guidance in its functions when needed. I also greatly appreciate the kindness you have shown in sharing your materials with me when I have been less thoughtful about preparing my own.

Thanks to Christoph Howe for your guidance in the use of confocal microscopy even though you were in the middle of writing your own licentiate thesis. Without you, my work would have been set back even further.

Thanks to Pia Lindberg for accepting to review my report and for providing answers and comments on some of the questions I have had throughout the project.

Special thanks go out to Karl Holdar, Angelika Vizintin, Giovanni Barone and Nikolas Spanoudakis for your close companionship over the last semester.

Thank you Claudia Durall de la Fuente and Feiyan Liang for always being able to bring a smile to my face no matter what the situation as well as providing necessary help.

Thanks to all other members of the cyano group at microbial chemistry Ångström, for all the help you have given me as well as the friendship you have shown towards me. You have all helped to make this project the so far most personally developing experience of my life.

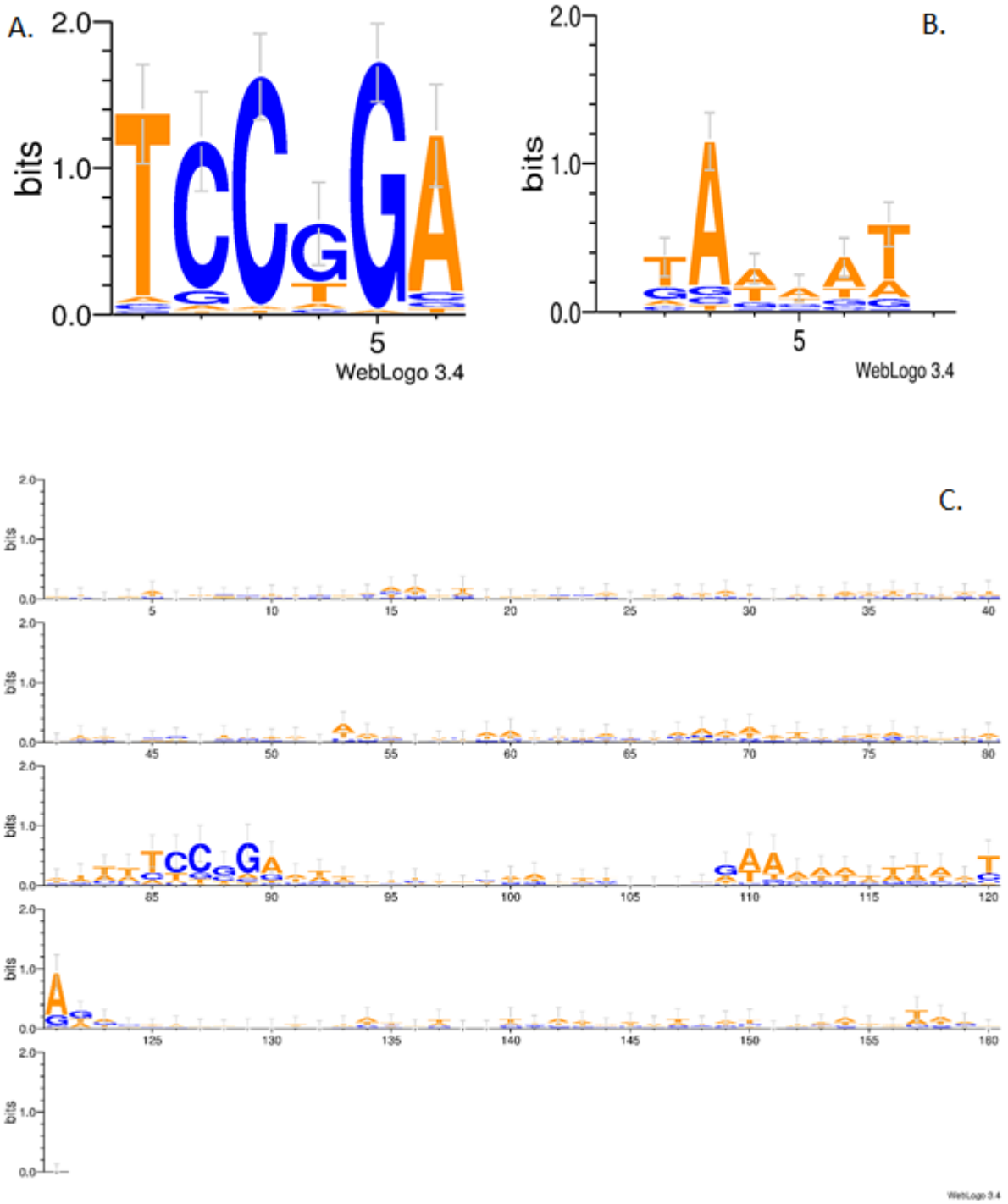
I would additionally like to thank my friends in the molecular biology engineering programme for all the help they have provided me with during these last 5 years. Especially this thanks goes out to Magnus Bergman, without your support I would most likely not be at this point today.

Thank you Anna Olofsson and Marcel Llavero Pasquina for proofreading and opposing my thesis.

Finally, I would like to thank my lovely girlfriend Hanna Eriksson who has put up with my behavior during the writing of this report as well as provided help and support with illustrations and proof reading.

Appendix A. Weblogo studies

This appendix features a weblogo study of the conserved regions present upstream of the NsiR I transcription start site. The top two picture shows conservation of the DIF motif in heterocyst-specific promoters (A) compared to the conservation of the regular pribnow-box in non-heterocyst-specific promoters (B). Picture C shows total sequence conservation of the upstream region through all investigated promoters.



Appendix B. BGII recipe

BGII and $BGII_0$ stock solution recipe

Stock No.	Stock solution components	(g/L) for 1000 x
Stock 1	K_2HPO_4	**40**
Stock 2	$MgSO_4 + 7\ H_2O$	**75**
Stock 3	$CaCl_2 + 2H_2O$ **Citric Acid**	**36** **6.0**
Stock 4	**Ferric ammonium citrate** **EDTA disodium salt**	**6.0** **1.0**
Stock 5	Na_2CO_3	**20**
Stock 6 (Trace metal mix)	H_3BO_3 $MnCl_2 + 4\ H_2O$ $ZnSO_4 + 2H_2O$ Na_2MoO_4 $CuSO_4 + 5\ H_2O$ $Co(NO_3)_2 + 5\ H_2O$	**2.86** **1.81** **0.222** **0.395** **0.0790** **0.0494**

- Add 1 mL of each of the stock to 1 L of distilled water to prepare $BGII_0$.
- To prepare BGII add 10 mL/L of $NaNO_3$ from a 100 x stock (150 g of $NaNO_3$ tp 1 L of distilled water).
- Each of the stock solutions can be prepared in bulk, sterilized and frozen in -20°C freezers. Working falcons of the same stock can be stored at 4°C.
- To prepare BGII agar add 1% agar to the media.

Appendix C. Important sequences

Displayed below is each individual promoter sequence with color coding as described in picture.

>Synthetic DIF-promoter (92 bp)

TATCGAGCTCTCTAGAACGCATTGTTTTTCCGGAGATAGGTAGATAGTTTAGAAAAATTTAATATAGGATCCTAGTG
GAGGTCTCGAGAGTA

> *NsiR1* core-promoter (92 bp)

TATCGAGCTCTCTAGAGTGCAGATTCATCCGGAATTGAGTGAATATGGATAGAACAATTAATTGTAGGATCCTAGT
GGAGGTCTCGAGAGTA

> *NsiR1* long-promoter (116 bp)

TATCGAGCTCTCTAGATGTTCATAAATAACCAGCATAATCGTGCAGATTCATCCGGAATTGAGTGAATATGGATAG
AACAATTAATTGTAGGATCCTAGTGGAGGTCTCGAGAGTA

>*NsiR1* mix-promoter (115 bp)

TATCGAGCTCTCTAGATGTTCATAAATAACCAGCATAATCGTGCAGATTCATCCGGAGATAGGTAGATAGTTTAGA
AAAATTTAATATAGGATCCTAGTGGAGGTCTCGAGAGTA

>*NsiR1* -10 -promoter (92 bp)

TATCGAGCTCTCTAGAGTGCAGATTCATCCGGAATTGAGTGAATATGGATATATAATGTGTGGGTAGGATCCTAGT
GGAGGTCTCGAGAGTA

This is followed by a description of the consensus sequence of the 12 chromosomal versions of the native NsiR I promoter as described by Muro-Pastor, 2014. Green underlining marks presence of the conserved motifs and yellow the full core sequence.

>*Conserved NsiR1 core*

5'GTAGATGCACCCTGATAACTAACTCCCCTAGCTGGCTAACACCGACTGGGGGCTTTTTTTATGTTCATAAATAACC
AGCATAATCGTGCAGATTCATCCGGAATTGAGTGAATATGGATAGAACAATTAATTGGTAGATGCACCCTGATAAC
TAACTCCCCTAGCTGGCTAACACCGACTGGGGGCTTTTTTATGTTCATAAATAACCAGCATCATT3'