# Development of a Fluorescent Repressor Operator System (FROS) Based on LexA for DNA Labeling in Live Cells

Research Thesis

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# 1 Abstract

Beyond the genetic information encoded in the linear DNA sequence, the spatial conformation of chromatin, namely the genome and its associate biomolecules, plays an important role in regulating a variety of processes in eukaryote cells, e.g., gene expression and regulation, DNA repair, nuclear transport, and more. DNA spatial organization in fixed cells is determined by various biochemical methods, such as Hi-C, while methods for tracking chromatin dynamics in live cells, such as fluorescence microscopy, are limited due to high acquisition time.

FROS (Fluorescent Repressor-Operator System) is a technique used to visualize DNA loci in live cells. In this method, an array of multiple repeats of protein-binding sites (operators) is integrated at a specific locus in the genome and is visualized by the specific binding of fluorescently tagged repressors. FROS is used in yeast to study chromatin dynamics, by tracking the positions of labeled DNA loci in time. For contextual information, it is often necessary to observe the positions of several different loci. For example, during mating-type switching event, there are four known main loci involved in the process simultaneously. However, only three FROS, based on repressor-operator of Lac, Tet and Lambda, have been used for the localization of three different loci involved in mating-type switching. This example emphasizes the need for developing additional FROS, as it enables the tagging of a larger number of specific loci, ultimately achieving better sampling of the genome and higher resolution of DNA dynamics. Moreover, optimization of signal intensity, which among others depends on repressor-operator interactions, is crucial for the usage of advanced microscopy methods such as PSF engineering, for fast 3D localization microscopy.

Here, we develop a new FROS using the bacterial LexA operator -repressor system together with iRFP720 fluorescent protein. This system uses fewer operator repeats compared to the other FROS, which reduces genome perturbations, with strong enough fluorescence intensity to allow extended tracking and enable challenging imaging modalities such as PSF engineering. We employed the new system, together with the widely used Tet system, to simultaneously visualize two different loci involved in chromatin reorganization associated with mating type switching in *Saccharomyces cerevisiae* yeast cells. This is a step towards high precision 3D dynamic visualization of at least four fluorescently labeled DNA loci at a single live cell level, using PSF engineering.

# 2 List of abbreviations

- PSF – Point Spread Function
- FROS - Fluorescent Repressor Operator System
- 3D – three Dimensions
- BB – Backbone
- HF – High Fidelity
- CC - Competent Cells
- O/N – Over night
- RT - room temperature
- bp - base pairs

# 3 Introduction

## 3.1 Genome spatial organization in the nucleus

The genetic information of eukaryote cells is stored in a complex structure called a chromosome, polymers of DNA in complex with other proteins such as histones, that are folded into a tight form[1]. The spatial configuration is found to be correlated with gene expression and regulation, replication timing, DNA repair and other cellular functions[2,3,4]. This Implies that chromatin spatial conformation is highly dynamic and continuously changes due to the physiological and transcriptional state of the cell[2]. Genome folding and misfolding inside the 3D nucleus is directly linked to genome dysfunction, leading to the onset and progression of a broad range of pathogenic phenotypes and diseases, such as limb malformation syndromes[5] and cancer[6]. There are still many unresolved questions regarding the spatiotemporal conformation of chromatin *in vivo*, and the most natural way to investigate DNA dynamics is to observe chromatin in 3D, in single, live cells.

### *3.1.1 Approaches to investigate the spatial configuration of DNA*

Different types of chromosome capture techniques (3C and its derivatives- 4C, 5C and Hi-C), and fluorescence *in situ* hybridization (FISH), provide crucial information regarding the three-dimension (3D) interactions between different loci in the nucleus of fixed cell population. Chromosome capture techniques provide inter-loci spatial proximity maps that reveal important interactions between genomic loci that may be located at a great distance in the linear DNA, by crosslinking the interactions between the loci or DNA-protein complexes using formaldehyde[7]. On the other hand, DNA FISH enables the measurement of 3D distances between a limited number of genomic loci using fluorescently labeled DNA probes to specific loci on the chromatin fiber[3]. While exposing unknown conformational characteristics, these techniques cannot provide dynamic information, hence dynamic features or transient unstable events that are only visible in a small fraction of cells cannot be revealed using these methods. Although combining information from those techniques with biophysical simulations obtain new insights into the large-scale

chromatin architecture, they still do not provide information about DNA dynamics in single live cells[8]. Conversely, fluorescence microscopy serves as an ideal tool to address different biological question related to spatiotemporal conformation *in vivo*, due to its biocompatibility, high specificity, and time resolution[9]. Fluorescence microscopy is an optical imaging technique that uses fluorescence to generate an image, by illuminating the sample that was pre-labeled with fluorophores or fluorescent proteins, and detecting the emitted light using an emission filter.

Fluorescent Fusion Proteins (FFP), which are used for *in vivo* fluorescence microscopy, are generated by the fusion of two genes, one encoding fluorescent protein and the second encoding a cellular protein (the protein of interest), with a linker that allows proper folding of the two translated proteins (each maintains its natural functionality). A protein of interest can then be monitored in live cells without having to purify, label, and deliver the protein into cells, enabling the measurement of cellular processes of interest in the native environment, by the visualization of the tagged protein with fluorescence microscopy[10]. The precise identification of specific chromosomal loci in living cells was initially rendered possible by the development of a green fluorescent protein (GFP)-tagged lac repressor–operator system for site recognition[11].

### *3.1.2 Labeling approaches to observe DNA dynamics in single live cell*

To track chromatin dynamics in 3D in a single live cell, specific and non-destructive techniques are required. A novel and promising method based on the CRISPR-Cas9 bacterial system has been recently developed, termed CRISPR-dCas9[12,13]. For fluorescent labeling purposes, rather than genome editing, a fluorescently labeled nuclease deficient Cas9 is used and the locus is labeled through specific binding of the programmed fused protein. An alternative labeling approach called CRISPR display[14], in which the gRNA is fluorescently labeled with an addition of single or multiple stem-loops that can be recognized by fluorescent loop-binding proteins. Although CRISPR-based methods are promising tools for genome editing, they suffer from off-target activity of the Cas9/dCas9 proteins, resulting in nonspecific labeling, and usually require multiple gRNA designs[15].

#### *3.1.2.1 Fluorescent Repressor Operator System (FROS)*

Fluorescent Repressor Operator System (FROS) is a technique in which an array (a repeated sequence) of a protein-binding site is integrated at specific loci in the genomic DNA and is visualized through the fluorescence of multiple copies of a bacterial repressor fused to a fluorescent protein, which specifically binds to each repeat in the array. Ideally, the result appears as a visible bright fluorescent spot over a dim background of diffusing unbound fluorescent proteins[2,16,17]. The insertion of the operator arrays, as well as the genes coding for the corresponding repressor proteins at specific sites in the yeast chromatin, is achieved using the efficient yeast transformation process. Due to high signal to noise ratio (SNR) and ease of integration into various sites in the genome, FROS has been used in yeast to study chromosome behavior in numerous studies on chromatin dynamics[11,18,19,20,21,3]. However, the use of FROS requires the insertion of large and repetitive DNA fragments, which may cause genome perturbation.

To address the need for multicolor spots to follow multiple loci, there are already three established systems of distinct operator-repressor specificities (LacO-LacI[11], TetO-TetR[22], and LambdaO-LambdaR[23]) that allow the localization of three fluorescence proteins with separate emission spectra at different loci on the yeast chromatin.

## 3.2 *Saccharomyces Cerevisiae* mating type switching

The budding yeast chromosomes are known to be located at distinct nucleus domains. Furthermore, specific loci within each chromosome are highly organized: dense/silenced chromatin (heterochromatin) tends to be localized near the nuclear periphery, whereas open/expressed chromatin (euchromatin) tends to be localized in the lumen of the nucleus[21,24]. *Saccharomyces Cerevisiae* is a haploid eukaryotic budding yeast having the euchromatin MAT locus located in the right arm of chromosome III, between the centromere and the telomere. This locus contains regulatory elements, promoters and encoding areas for proteins involved in different sexual appearances: MATa or MATα as haploids, and MATa/MATα as diploid following haploids conjugation. In addition to the MAT locus, chromosome III has two silenced, unexpressed, loci encoding for the mating type genes: HMLα and HMRa (Figure 1). Both sequences are strongly silenced by *cis*-acting elements and *trans*-acting factors and are normally protected from cleavage by a restriction enzyme such as HO endonuclease[24,25,26].

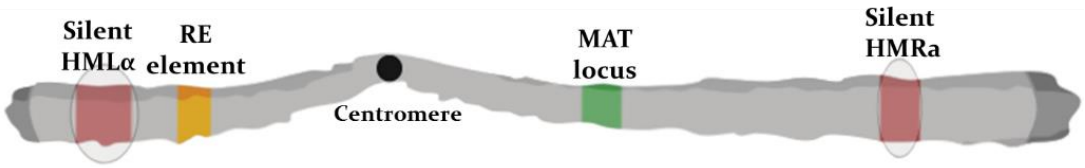



**Figure 1: Mating type switching loci on chromosome III in *S. cerevisiae*.** MAT locus (green), Silent HMRa & HMLα (red) and recombinase enhancer (RE) (yellow).

Homothallic mating type switching occurs when one haploid mating type (α / a) switches to the opposite type. The process is initiated upon double-strand-break (DSB) at a 24-bp sequence in the mating type-specific locus within the MAT locus by HO endonuclease. The break is then repaired by gene conversion (a type of HR repair pathway) using one of the aforementioned preferred donors (HMLα/ HMRa) sequence, which remains unchanged following switching (Figure 2)[24,25,26].

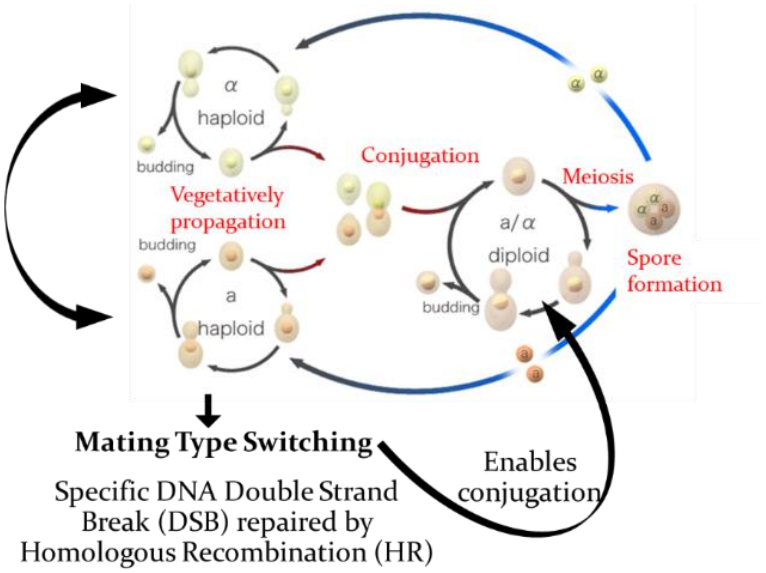



**Figure 2: *Saccharomyces cerevisiae* life cycle.** Yeast cells can divide as haploids or diploids. To maintain equal numbers of a and α mate types in the haploid population, the mother cell undergoes switching to the opposite type in the G1 cell cycle phase. Haploid a and α cells can conjugate and propagate as diploids, which can then undergo meiosis back to haploids.

During the mating type switching event, donor selection is highly directional. A DSB at MATa/ MATα locus is followed by gene-conversion repair using a sequence found at the unchanged donor HMLα/ HMRa locus in 85-90%/90% of the times, respectively, and switch to the opposite cell type[24,27,28]. In addition to the three relevant MAT switching loci, the left arm of chromosome III contains a recombination enhancer (RE) region (Figure 1), which is composed of highly conserved elements and protein binding sites. This locus is found to be an enhancer for the mating type switching for MATa cells by mediating HMLα toward MATa, resolving partially the question of how HMLα serves as a donor for MATa when HMRa proximity is twice smaller in base pairs (bp)[25,24,28,29].

Mating-type switching is an important model system often used to study the mechanism of homologous recombination after DNA double strand break (DSB) repair[18,30]. Importantly, the process involves a dynamic conformational change of DNA with at least four well-defined DNA loci. To control the timing of the process and to allow it to occur in parallel in the entire cell population, knockout yeast cells for HO endonuclease are used and integrated with a plasmid containing the HO gene under the control of the GAL promoter, allowing regulated induction of the DSB at MAT locus upon galactose addition[31,18].

## 3.3 3D localization microscopy by Point-Spread-Function (PSF) engineering in living cells

Cellular processes that control the most critical aspects of life occur in three-dimensions (3D) and are intrinsically dynamic. Tracking of a single object (localization) from a series of sequential images (frames) yields the trajectory of the moving object. This spatiotemporal trajectory can provide information on different physical properties such as particle size, conformation, and the local environment. The 3D trajectory of a moving particle can be extracted in several ways, such as scanning methods[13], the most popular of which being confocal scanning which achieves Z-sectioning and good lateral resolution[32,33,34] but requires a long acquisition time. Other techniques are widefield microscopy that is capable of faster image acquisition but provides lower spatial resolution compared to the scanning-based techniques[35], and stochastic-labeling imaging that achieves high spatial resolution- 10 times better than the diffraction limit, but it inherently slow and mostly incompatible with live imaging[36,37,38,39,40].

Due to the inherently limited temporal resolution of scanning-based imaging methods, over the years, various scan-free methods for 3D particle tracking have been developed. Among them are the Point-Spread-Function (PSF) engineering approach, in which the depth of a point source is encoded in the shape it creates on the camera due to interaction with an optical element- the phase mask[41,42,43,44].

In standard fluorescence microscopy, each in-focus illuminated point in the sample emits light which is collected in the image plane as a shape of an airy disk, enabling the determination of the lateral position ($X$, $Y$) of a point source by finding the center of the PSF. However, this method is inherently limited in the axial ($Z$) direction. In focus, the emitter is seen as a bright dot, but as the emitter goes above or below the focal plane, it becomes blurry and quickly cannot be distinguished at all. An additional challenge is introduced when visualizing multiple emitters (in one or more colors). Emitters in close proximity (below resolution limit) cannot be distinguished, thus seen together as a single blur[45] (Figure 3).

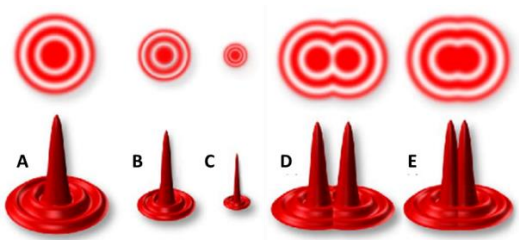
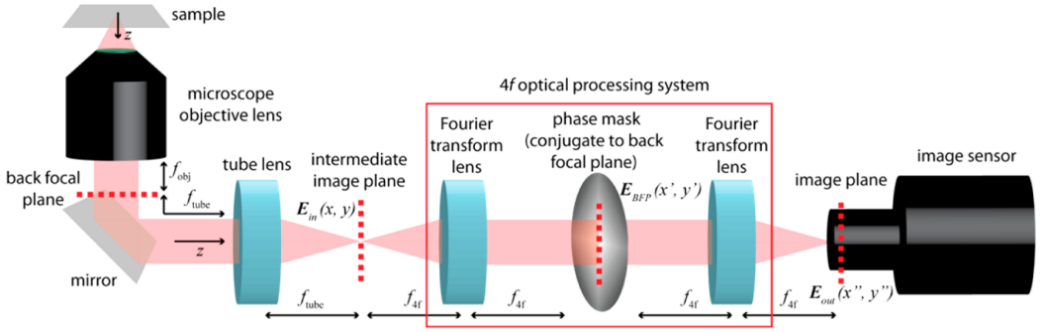



**Figure 3: Airy disk PSF illustration**. The Airy disk size depends on the objective numerical aperture (NA). (A) wide profile due to low NA; (B) moderately wide profile due to moderate NA; (C) narrow profile due to high NA; (D) Airy disk intensity profile for emitters in the limit of resolution; (E) PSF for emitters in very close proximity with overlapping center, obtains single unresolved localization

Point-Spread-Function (PSF) engineering overcomes the limited axial range problem. Implementation is based on a standard inverted microscope modified with an added *4f* optical system (Figure 4) after the standard image plane. The modification includes a lens placed in the first focal plane which projects a Fourier transform onto a second focal plane. In this plane, namely the Fourier plane, a phase mask that modulates the wavefront is placed. The purpose of the phase mask is to modify the wavefront of the emitted fluorescent light in a way that is sensitive to the depth of the emitter. The modulated wavefront undergoes inverse Fourier transform due to the lens placed in a third focal plane, which translates to the modulated image onto the camera focal plane[42,43].

**A**




**B**

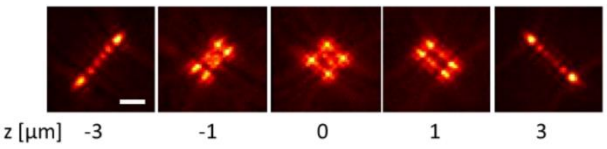



**Figure 4: Schematic illustration of *4f* optical system. A.** Conventional inverted fluorescence microscope is modified by adding optical elements in 4 focal planes: lens in the 1st plane, dielectric phase mask in the 2nd (Fourier) plane, additional lens in the 3rd plane and camera in the 4th (image) plane[43]. **B.** Measurement of a 200 nm fluorescent microsphere in various depths over a 6 μm range, imaged using the Tetrapod PSF. Extracting the 3D position from the PSF is done by fitting the image to a calibrated PSF model. Scale bar = 2 μm.

The output of this optical setup is a widefield 2D image, in which a modified PSF is detected on the camera when imaging a point source, such that the shape encodes for the accurate *X*, *Y*, and *Z* (3D) positions for each imaged emitter. The specific phase mask that we use, yielding the Tetrapod PSF, is an optimally precise PSF for depth determination, tailored to the imaging system[42,46]. Such a PSF, exhibits a variable applicable depth range, capable of increasing the depth of field of the microscope by a factor of ~forty[42]. By either adding multiple phase masks (one per color channel) or using a multicolor mask [ref Shechtman 2016], this method enables fast (scan-free) 3D tracking of multiple DNA loci tagged with different fluorophores, observing their dynamic simultaneously (in the single live cell level), with very short acquisition time (tens ms scale) and high spatial resolution (tens of nm scale in x,y,z; and optimally ~6μm range in the axial direction, for an oil immersion 100X objective, under live-cell conditions)[41,47,48].

# 4 Research objectives

The overall aim of this study was to provide a new FROS using the bacterial LexA repressor-operator system with iRFP720 fluorescent protein. Developing additional FROS is highly desirable, as it enables tagging more loci, each with a different fluorescent proteins and separate emission spectra. I demonstrated the applicability of the new FROS by labeling two loci involved in mating-type switching event for future evaluation localization throughout the process, and for imaging and tracking their 3D trajectories using PSF engineering. The latter will allow for increased understanding of chromosome III spatial organization in the nucleus as part of the global chromatin reorganization, position and behavior.

## 4.1 Specific aims

1. Constructing a plasmid containing an appropriate number of LexA operator repeats that can be integrated into the yeast genome and visualized upon binding of LexA repressor conjugated to a fluorescent protein,
2. Constructing a plasmid containing a gene that constitutively encodes nuclear localized LexA repressor fused to iRFP720 fluorescent protein.
3. Integrating the new FROS into W303-B yeast cells and characterizing its labeling efficiency.
4. Integration of LexA and Tet system simultaneously at two loci involved in mating-type switching event- HMRa and HMLα, respectively.
5. Testing the specificity of the recognition of each repressor to its operator sequence by integrating different unmatched combinations of repressor/operator pairs of LexA, Tet and Lac systems.
6. Demonstrating the photon-demanding application of 3D imaging and tracking of a LexA fluorescent spot via PSF engineering.

# 5 Results

## 5.1 Construction of new LexA FROS

### *5.1.1 LexA operator/repressor system*

LexA repressor is naturally involved in bacterial SOS response for DNA damage. When bound, LexA represses the expression of a set of SOS response genes coding for different proteins and inhibitors, such as DNA repair enzymes and error-prone DNA polymerases. Upon DNA damage, the repressor cleaves itself in a process called autoproteolysis, enabling the genes expression[49]. However, the LexA repressor does not take part in any regulation response in yeast cells, thus its binding to the LexA operator can be used for labeling purposes[50,51].

To test whether the new LexA operator/repressor system can be used for specific locus labeling for the first time, in W303-1B *saccharomyces cerevisiae* yeast strain (YA204), the LexA repressor fused to iRFP720 fluorescent protein was expressed under the control of HIS3 promoter. Prior to LexA operator array integration, fluorescent cells were visualized all over the cell using an epi-fluorescent microscope (Figure 5A, upper). Next, an array of approximately 64 repeats of LexA operator, generated by multiple cycles of restriction and ligation, was integrated at URA3 locus in chromosome V, resulting in a bright spot in the nucleus (Figure 5A, lower). This result showed a successful DNA labeling using LexA system after the integration of LexA operator array, which was not possible in the absence of the array or by any protein that is naturally expressed in yeast cells. However, the integration did not confirm by genomic sequencing.

Since LexA is a bacterial repressor it does not naturally fuse to a nuclear localization signal (NLS) and can therefore exit and enter the nucleus of yeast cells spontaneously. In an attempt to localize the proteins in the nucleus, we fused the SV40 short peptide ($PK_3RKV$) to the repressor encoding sequence at the C- terminus, resulting in enhanced fluorescence localized spot signal in the nucleus and significantly lower signal all over the cell, which enhanced the signal-to-background ratio (average ratio of 1.83 with the addition of NLS compared to average ratio of 1.37 before the fusion of the peptide), an important parameter for super resolution microscopy and in particular for PSF engineering (Figure 5B).

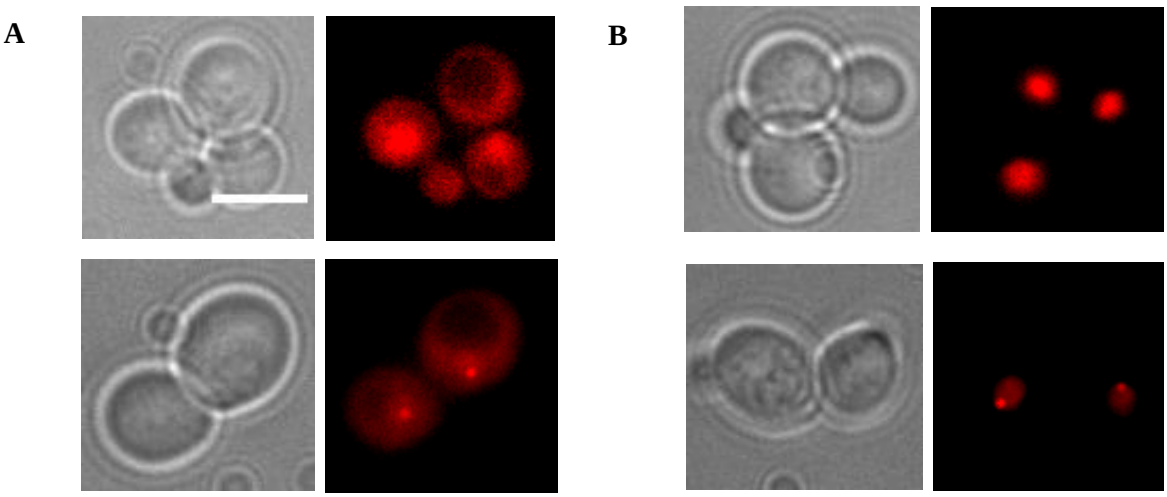



**Figure 5: DNA labeling using LexA system and the influence of NLS on the localized fluorescence signal.** Yeast cells in a logarithmic phase in SC-LFM (2% glucose) growth medium were visualized under an inverted fluorescent microscope **A.** Left- brightfield images, right- yeast cells expressing LexA binding protein fused to iRFP720 fluorescent protein under the control of HIS3 promoter (upper), and a localized spot in the nucleus as a result of LexA operator array integration into URA3 locus (lower). **B.** Left- brightfield images, right- yeast cells expressing iRFP720-LexA binding protein with the addition of SV40 signa (upper), and a localized spot as a result of LexA operator array integration into URA3 locus (lower). Scale bar- 5μm.

In previous studies, two (MAT and HMLα, HMRa or RE)[52,27] and three (HMLα, MAT and HMRa)[23,53,27,3] different loci involved in mating-type switching were labeled simultaneously with a combination of Tet, Lac and Lambda systems, each uses a repressor fused to different fluorescent protein with separate emission spectra. To lay the basis for labeling four different loci simultaneously with the new labeling system, the LexA system with iRFP720 fluorescent protein was integrated by homologous recombination at HMRa region (ChrIII:295192-295337) and the well-established Tet system with mCherry fluorescent protein was integrated at HMLα region (ChrIII:15088-15621). The two distinct labels were clearly visualized and were spatially separated, however only a small subset out of the entire population (less than 1%) were labeled in both loci (more than 95% of the cells were labeled with the Tet system) (Figure 6).

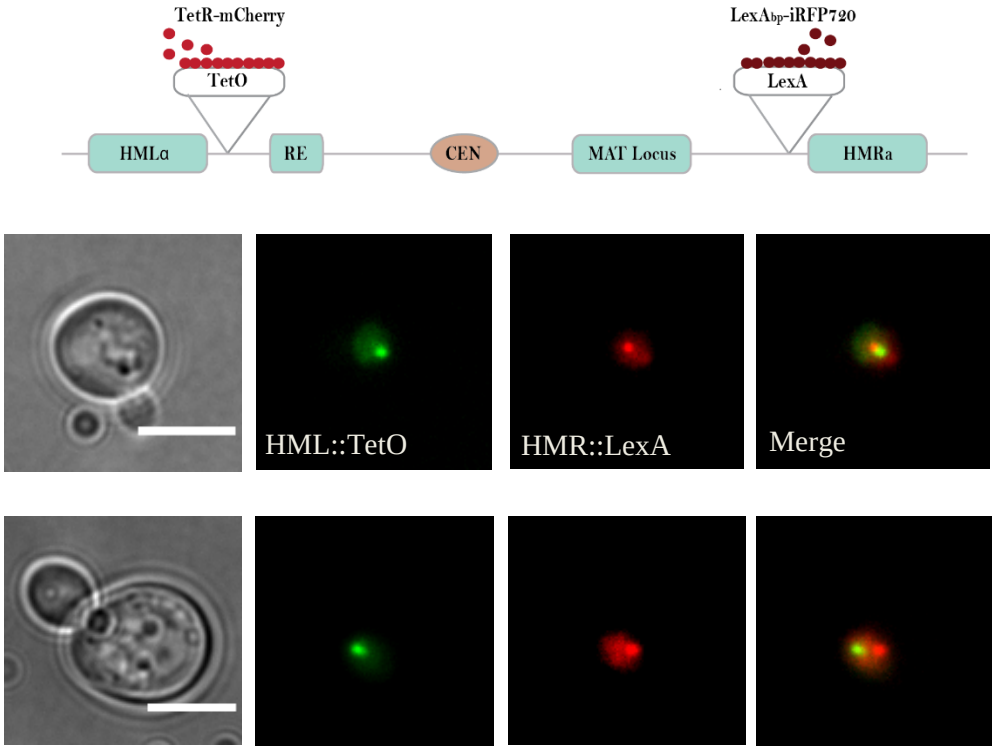



**Figure 6: Labeling two loci involved in mating-type switching event (HMLα and HMRa).** Yeast cells in a logarithmic phase in SC-LFM (2% glucose) growth medium were visualized under an inverted fluorescent microscope. Representative brightfield (left) and fluorescent images of localized LacI-mCherry and LexA-iRFP720 at HML and HMR on chromosome III. Insertion sites of LacO and LexA arrays are indicate. Scale bar- 5µm.

### *5.1.2 Comparing fluorescence intensities of localized spots obtained from LexA, Tet, Lac and Lambda systems*

High fluorescence intensity of the localized spot is crucial for high precision localization, in particular for PSF engineering purposes. The spot fluorescence intensities formed by LexA, Tet and Lac systems were compared during successive imaging in the center of the field of view by integrating the plasmids containing genes encoding different repressors fused to a fluorescent protein under His3 promoter, and the plasmids containing arrays of operator repeats under the control of Ura3 promoter. However, Lambda system did not form any localized spot (Figure 7).

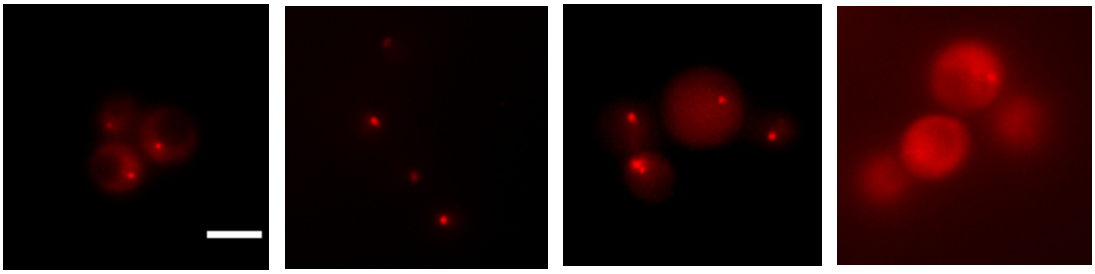


**Figure 7: Localized spots generated from LexA, Tet, Lac and Lambda systems.** Yeast cells in a logarithmic phase in SC-LFM (2% glucose) growth medium were visualized under an inverted fluorescent microscope. Integration of LexA binding sites, TetO, LacO and LambdaO at Chr. V into yeast cells expressing LexA binding protein, TetR, LacI and Lambda cI fused to iRFP720 fluorescent protein, respectively. Scale bar- 5µm.

The localized spot fluorescence intensity obtained from LexA system was the strongest and remained stable compared to Tet and Lac systems (average fluorescence of 18799 for LexA compared to 13910 for Tet and 11924 for Lac at starting time), even though LexA uses fewer operator repeats. However, the background fluorescence intensity of the new LexA FROS was the highest compare to the background intensity obtained from Lac and Tet systems (average of 8076, 2637 and 3021 respectively), thus the signal to background ratio of the new FROS was the lowest (2.33 for LexA, 5.27 for Tet and 3.94 for Lac) (Figure 8).

The ratio between the fluorescence signal of a localized spot to the fluorescence signal of the background due to unbound fused fluorescent repressors was calculated by measuring the mean values of fluorescence intensity within a squared boundary that was defined ones for the localized spot and ones for the smeared fluorescence within the nucleus (Equation 1). Values were taken from FiJi software using the "measure" plugin.

$$SBR = \frac{Average\ localized\ spot\ fluorescence\ intensity}{Average\ backgound\ fluorescence\ intensity}$$

**Equation 1.** Signal to background fluorescence intensity ratio calculation

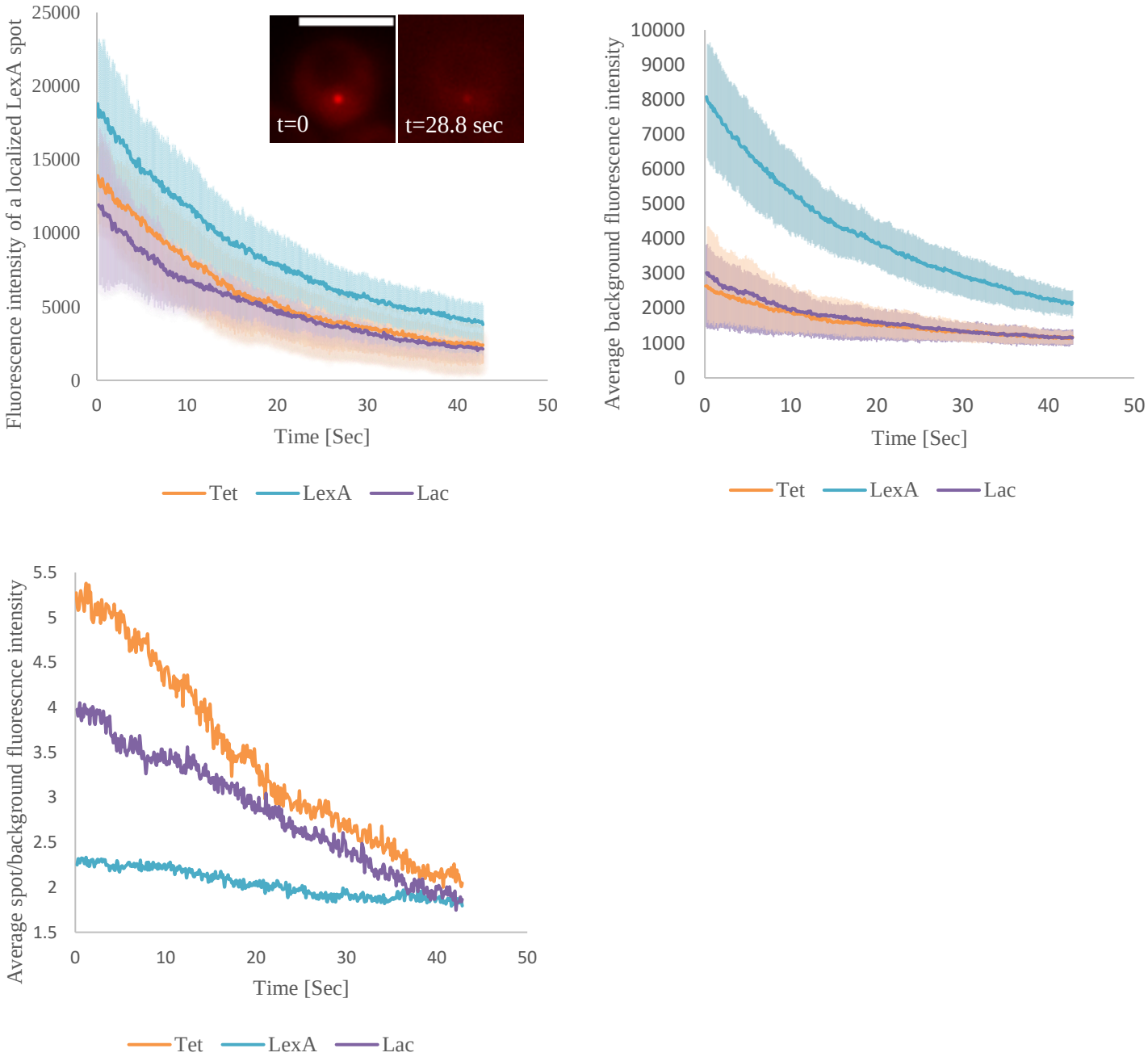



**Figure 8: Fluorescence intensities of a localized LexA spots, background and average spot/background formed by Tet, Lac or LexA systems during imaging.** Tet, Lac and LexA systems with iRFP720 fluorescent protein were integrated into yeast cells and the fluorescence intensity of the localized spot, background and spot signal/background ratio was measured during time. Presentative fixed yeast cell bearing localized LexA spot is presented in time 0 and after 28.8 second of imaging. Scale bar- 5µm.

### *5.1.3 Orthogonality between different repressor/operator combinations*

To label multiple loci simultaneously with different FROS, specificity and high affinity of each repressor to its operator are among the most important parameters to allow precise labeling and high signal. To test whether LexA repressor is highly specific to its operator, and that the LexA operator cannot be recognized by other proteins, different combinations of unmatched repressor/operator array were introduced to W303-1B (YA204) yeast cells. The fluorescence with all unmatched combinations was visualized all over the nucleus, meaning that LexA repressor binding is highly specific to LexA operator.

Different plasmids containing genes that encoding repressors (either LacI, TetR or LexA repressor) fused to iRFP720 fluorescent protein were integrated at His3 locus in YA204 cells, and cells with the highest fluorescence intensity at the nucleus as visualized under fluorescence microscope were selected. Then, the selected cells were integrated with a plasmid containing an unmatched operator array (either LacO, TetO or LexA operator). None of the unmatched combinations formed a localized spot (Figure 9), which means that the recognition of each repressor to its operator is highly specific, and the three systems- Lac, Tet and the new LexA can be used simultaneously to label three different loci in live yeast cells.

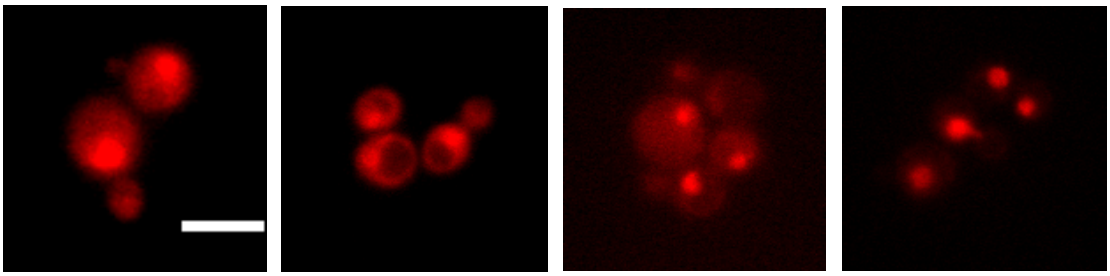


**Figure 9: Yeast cells integrated with different repressor/operator combinations to test orthogonality.** Yeast cells in a logarithmic phase in SC-LFM (2% glucose) growth medium were visualized under an inverted fluorescent microscope. Different combinations were examined to verify specificity and unique recognition of each repressor to its compatible operator. Integration of LacO and TetO into yeast cells expressing LexA binding protein, and integration of LexA binding sites into yeast cells expressing LacI and TetR at Chr. V. Scale bar- 5µm.

### *5.1.4 3D tracking of LexA labeled locus using PSF engineering*

PSF engineering modifies the image forms on the detector into a new shape, known as Tetrapod PSF, efficiently encoding the depth of the point source in its shape[42,48]. When light is collected from a point source (e.g. fluorescently labeled DNA locus by LexA FROS), it interacts with a phase mask, an optical element that is added to the optical path, and encoding additional information contained in the phase onto the resulting image. To test whether the new LexA system is suitable for this purpose, i.e the signal-to-background of the labeled locus is high enough for super resolution imaging, we added a dielectric phase mask (as described in section 6.7) to the

optical path and were imaged the localized spot at URA3 locus in different Z positions (Figure 10). The tetrapod shapes were clearly visible and can be used for further localization analysis.

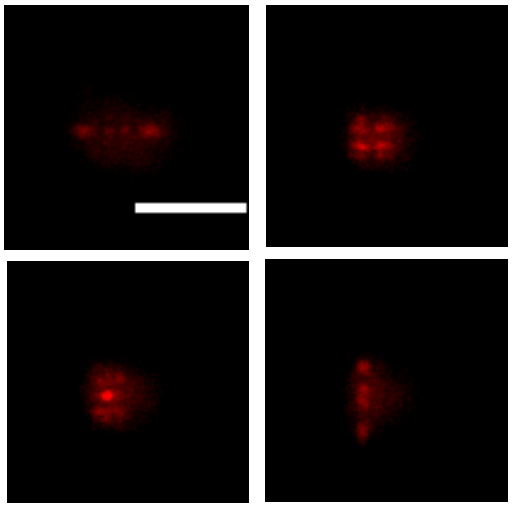


**Figure 10: Tetrapod PSF with the addition of dielectric phase mask to the optical path.** Yeast cells in a logarithmic phase in SC-LFM (2% glucose) growth medium were visualized under an inverted fluorescent microscope with a dielectric phase mask. Localized tetrapod PSF in different Z positions in YS0042 yeast strain bearing the LexA system with iRFP720 fluorescent protein at URA3 locus. Each PSF shape encodes the depth of the localized spot. Scale bar- 5µm.

The 3D trajectory can be measured then out of sequenced 2D images using the extended optical system with a dielectric phase mask (Figure 11).

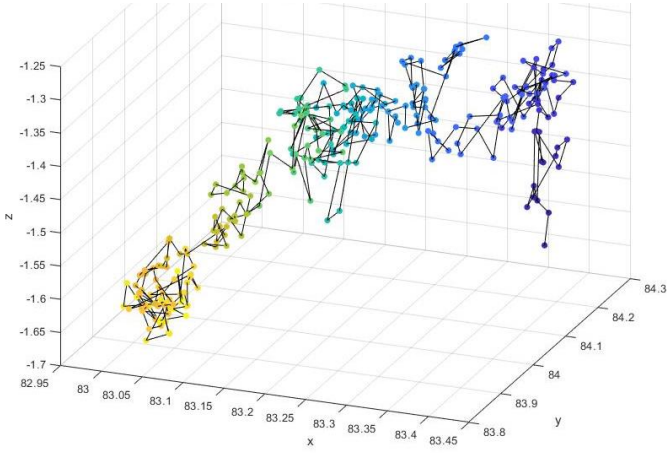



**Figure 11: 3D trajectory of a localized LexA spot**. Sequenced 2D images were used to measure the 3D trajectory in yeast cells bearing a localized LexA spot with iRFP720 fluorescent protein at URA3 locus. The colors changing from bright to dark during time.

### *5.1.5 Construction of LexA operator array using a library of LexA sequences*

In an attempt to reduce the repetitiveness of the array, and to stabilize the plasmid so it will be compatible for both bacteria and yeast growth, a library consists of short 20 base pairs oligos (ordered from IDT) containing 4 different LexA operator library each was designed[54,55,56]. Each 20bp binding site was served as an autonomous library, so that the bases flanking the sequence are consensus and the spacers between them vary by using IUPAC codes (Table 32). The spacers between each binding site are either 13N or 18N (which means 13 or 18 random bases), considering two factors. First, the protected region of the LexA repressor, that is, the sequence that the protein "covers" when binds to the operator[57]. The spacers should be long enough to allow two proteins to bind to adjacent operators. Second, in-phase and out-of-phase states of the operators. In the case of 18N spacer, two adjacent operators direct to the opposite direction, while in the case of 13N spacer the operators turn in the same direction. The single oligo sequences were assembled using different pairs of restriction enzymes with compatible ends, which when ligated generated a site that cannot recut with either enzyme, as diagrammed in Figure 13. The first ligation product generated 16 LexA operator repeats, which served as a template for the next round of restriction and ligation.

**Table 1:** Four LexA operator libraries

| Library name | DNA sequence |
|---|---|
| Type 1 | **TACTGT**WWWWWYW**TACAGTA** |
| Type 2 | **TACTGT**NWWWNTA**TACAGTA** |
| Type 3 | **TACTGT**ATRWDYA**TACAGTA** |
| Type 4 | **TACTGT**NWAHDHA**TACAGTA** |

According to IUPAC code, W=A/T, Y=T/C, R=G/A, D=G/A/T, H=A/C/T, N=G/A/T/C. https://genome.ucsc.edu/goldenPath/help/iupac.html

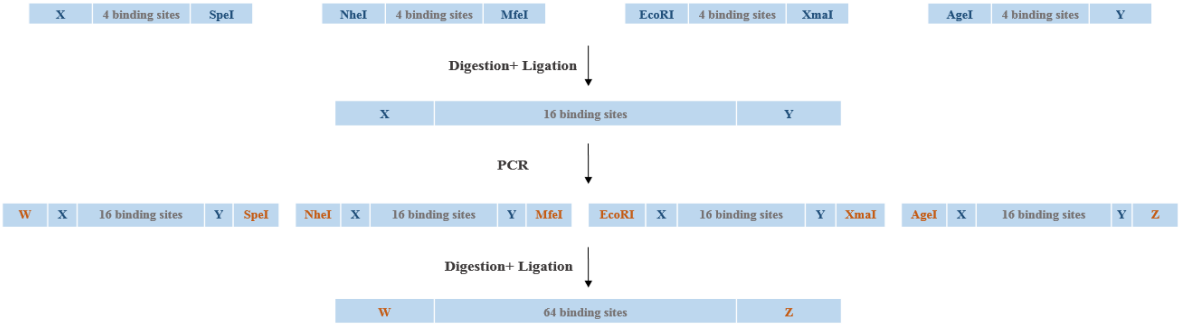



**Figure 12: Oligo assembly workflow**. Starting with 4 oligos containing 4 different LexA binding sites each, the oligos are assembled by restriction digestion with different combinations of restriction enzymes which form compatible sticky ends that after ligation creates a restriction site that cannot be recognized by either enzyme. The spacers between each binding site are either 13N or 18N (13 or 18 random bases). After the first round of restriction and ligation, the ligated product should contain 16 different binding site repeats, which serves as a template for the second round of restriction and ligation, which should then generate 64 LexA binding sites.

The results of the first round are shown in the gel image below (Figure 13). The expected length of 745bp received, together with additional unexpected bands.

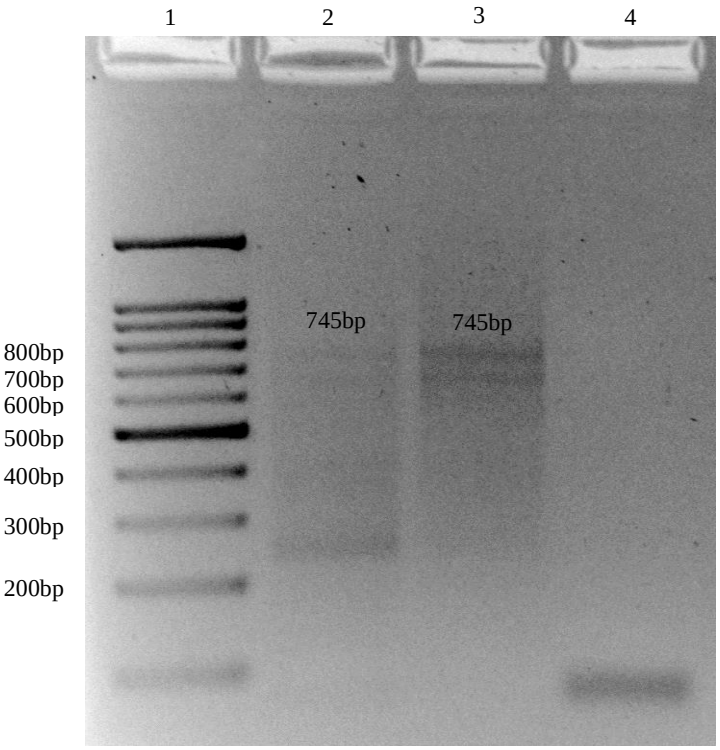



**Figure 13: PCR amplification of ligated product**. The first-round ligation product was amplified by PCR using specific primers for X and Y regions (Figure 12). Lane 1- 100bp ladder (DM 2100, SMOBIO), Lane 2- first round ligation product (50ng from each digested oligo), Lane 3- first round ligation product (100ng from each digested oligo), Lane 4- negative control (primers only).

However, the second round of restriction digestion and ligation did not double the number of the LexA binding sites. After a few attempts to repeat the second round without any success, this approach was abandoned since 16 binding site repeats are not enough to produce high fluorescence localized spot signal.

# 6 Methods

## 6.1 Polymerase Chain Reaction (PCR)

### *6.1.1 Extension of DNA fragments*

DNA fragments were amplified by PCR reaction from either genomic DNA or plasmid template. Each reaction added either restriction or overlap sites at the ends of the fragment for restriction-based method or for Gibson assembly reaction, respectively. After each PCR reaction, a sample of 1-5µl was run in 1-2% agarose gel for size validation. Verified PCR products were clean using PCR cleanup kit. Table 2 and 3 list the reaction materials and the PCR program, based on the commercial enzyme Q5 High-Fidelity DNA Polymerase.

**Table 2:** PCR materials

| Component | Volume[µl] |
|---|---|
| Q5 High GC Enhancer (5X) - optional | 10 |
| Q5 Reaction Buffer (5X) | 10 |
| dNTPs (10 mM) | 1 |
| Forward primer (10µM) | 2.5 |
| Reverse primer (10µM) | 2.5 |
| Template* | 10 |
| Q5 High-Fidelity DNA Polymerase** | 0.5 |
| MBW | 13.5 |
| Total | 50 |

*~1ng/µl for plasmid; ~10ng/µl for genomic DNA; **Add last

**Table 3:** PCR program

| Stage | Temp [$^0$C] | Time [sec] |
|---|---|---|
| Initial denaturation | 98 | 30 |
| 5 cycles | 98 | 10 |
| | 50-72* (short primer $T_m$) | 30 |
| | 72 | 20-30 Sec/Kb |
| 30 cycles | 98 | 10 |
| | 50-72* (long primer $T_m$) | 30 |
| | 72 | 20-30 sec/Kb |
| Final extension | 72 | 2 min |
| Hold | 4 | ∞ |

* The $T_m$ was determined using NEB $T_m$ calculator tool

### *6.1.2 Colony PCR*

#### *6.1.2.1 Bacterial colony PCR*

Initial screening of bacterial colonies was done using PCR with Taq ready mix (x2). The suspected colonies are added to the PCR mix and lysed during the initial heating step, causing the release of the plasmid which serves as a template for the PCR reaction. The reaction mix and the PCR program are described in Tables 4 and 5. For each reaction the appropriate primers were added, and the reaction products were run on 1-2% agarose gel for size verification.

**Table 4:** Bacterial colony PCR reaction mix

| **Ingredient** | **Volume for Taq ready mix (x2) [µl]** |
|---|---|
| Forward primer | 2 |
| Reverse primer | 2 |
| Template (picking isolated colony) | Single colony |
| Ready Mix for PCR | 10 |
| UPW / MBW | 6 |
| Total | 20 |

**Table 5:** Bacterial colony PCR program

| **Stage** | **Temp [$^0$C]** | **Time** | **Cycles** |
|---|---|---|---|
| Initial denaturation | 94 | 3 min | 1 |
| Denaturation annealing | 94 | 30 sec | 35 |
| | 55-70 (primer Tm) | 30 sec | |
| | 72 | 30 sec/Kb | |
| Final extension | 72 | 10 min | 1 |
| Hold | 10 | ∞ | |

#### *6.1.2.2 Yeast colony PCR*

Initial screening of yeast colonies was done using PCR with Phire plant direct PCR mix (x2). The reaction starts with a heating step to release the genomic DNA, which serves as a template for the PCR reaction. The reaction mix and the PCR program are described in Tables 6 and 7. For each reaction, the appropriate primers were added, and the reaction products were run on 1-2% agarose gel for size verification.

**Table 6:** Yeast colony PCR reaction mix

| **Ingredient** | **Volume for phire plant direct PCR mix (x2) [µl]** |
|---|---|
| Forward primer | 1 |
| Reverse primer | 1 |
| Template | Single colony |
| Ready Mix for PCR | 10 |
| UPW / MBW | 8 |
| Total | 20 |

**Table 7:** Yeast colony PCR program

| **Cycle step** | **2 step protocol** | | **3 step protocol** | | **Cycles** |
|---|---|---|---|---|---|
| | **Temp. [°C]** | **Time** | **Temp.** | **Time** | |
| Initial denaturation | 98 | 5 min | 98 | 5 min | 1 |
| Denaturation annealing | 98 | 5 sec | 98 | 5 sec | 40 |
| | - | | X* | 5 sec | |
| | 72 | 20 sec ≤1kb<br>20 sec/kb>1kb | 72 | 20 sec ≤1kb<br>20 sec/kb>1kb | |
| Final extension | 72 | 1 min | 72 | 1 min | 1 |
| | 4 | Hold | 4 | Hold | |

*Tm should be determined according to Thermo-Fisher Tm calculator tool: www.thermoscientific.com/pcrwebtools

## 6.2 Cloning

The initial cloning step includes PCR amplification of DNA fragments, using the *Saccharomyces Cerevisiae* genome or different plasmids backbone as templates, followed by cleaning using **NucleoSpin Gel and PCR Clean-up** kit and size verification using an agarose gel. Next, these amplicons were inserted into the plasmid backbone by either restriction & ligation or by Gibson assembly reaction.

### *6.2.1 Restriction and Ligation*

Amplified and cleaned DNA fragments and the plasmid backbone (BB) were digested by appropriate restriction enzymes (Table 8). Restricted fragments and plasmid BB were cleaned using **NucleoSpin Gel and PCR Clean-up** and ligated using T4 DNA ligase (Table 9).

**Table 8:** Restriction reaction mix

| Product | Volume [µl] |
|---|---|
| Enzyme Buffer (x10) | 2 |
| Restriction Enzyme | 1µl for 1000ng |
| DNA fragment | ~10 |
| Molecular Biology Water (MBW) | Adjust to a final volume of 20µl |

The time and temperature for each reaction are determined according to the restriction enzyme. For most of the reactions, the program was: 60min of digestion in 37˚C, followed by 20min of inactivation in 65˚C / 80˚C.

**Table 9:** Ligation reaction mix

| Product | Volume [µl] |
|---|---|
| T4 DNA ligase buffer (x10) | 2 |
| T4 DNA ligase (Enzyme) | 1 |
| DNA inset fragment | According to calculation done in http://nebiocalculator.neb.com/#!/ligation |
| DNA BB fragment | According to calculation done in http://nebiocalculator.neb.com/#!/ligation |
| UPW/MBW | Adjust to final volume of 20µl |

The ligation reaction tubes were incubated for 18 hours (O/N) at 16˚C, or for 1 hour at 30ºC, followed by 10 min of inactivation at 65˚C. The ligation products were transformed into competent cells (DH5α or Stbl2) and plated on an appropriate antibiotic plate.

### *6.2.2 Gibson assembly reaction*

In Gibson reaction[58], the amplified and cleaned DNA fragments (inserts and plasmid BB) undergo resection by T5 Exonuclease, enables the compatible ends to anneal, followed by a correction of the resected DNA by Phusion HF DNA polymerase, and nicked strand correction by Taq ligase.

A tube with Gibson master mix, either from NEB or the homemade, is thawed on ice for few minutes. Inserts DNA and plasmid BB are then added to the tube according to the follow calculation (Equation 2) and Tables 10 and 11.

- DNA concentration is measured in [ng/µl], and then transfer to [pmol/µl] using the online tool http://nebiocalculator.neb.com/#!/dsdnaamt.

Or by **Equation 2**: $\left[\frac{pmol}{\mu l}\right] = \frac{\left[\frac{ng}{\mu l}\right] \times 10^3}{fragment\_length_{bp} \times 650}$

*The molecular weight of a single DNA base-pair is 650 $\frac{gr}{mol}$

**Table 10:** Gibson assembly calculation

| | **Length [bp]** | **Initial concentration [ng/µl]** | **Initial concentration [pmol/µl]** | **3:1 ratio (Up to 3 fragments)** | **5:1 ratio (Up to 5 fragments)** |
|---|---|---|---|---|---|
| **Insert** | X | A | $\frac{A * 1000}{X * 650}$ | 0.15 | 0.175 |
| **Plasmid BB** | Y | B | $\frac{B * 1000}{Y * 650}$ | 0.05 | 0.035 |

- Each DNA fragment is diluted to receive in 1µl the request amount of DNA in pmol
- Ratio between each insert to BB is 3:1 or 5:1 (depend on number of inserts and fragments length)
- Total amount of all DNA fragments (inserts and BB) in each reaction is up to 0.5pmol

**Table 11:** Gibson assembly reaction

| | **Gibson master mix (x2) NEB [µl]** | **Gibson master mix Home-made [µl]** |
|---|---|---|
| Gibson Assembly Master Mix | 10 | 15 |
| Plasmid BB | 1 | 1 |
| Insert | 1 from each fragment | 1 from each fragment |
| MBW | Adjust to final volume of 20µl | Adjust to final volume of 20µl |

The Gibson reaction tubes were incubated at 50˚C for 1hr. The reaction products were transformed into competent cells (DH5α or Stbl2) and plated on an appropriate antibiotic plate.

## 6.3 DNA manipulation verification

### *6.3.1 Agarose gel electrophoresis for DNA separation*

Following PCR reaction, DNA fragments were separated according to their size using 1-2% agarose gel (Table 12) with TAE buffer (Table 13) for size validation. Ethidium-bromide (Et-Br) was added to the gel (1 drop per 50ml) to enable visualization of the DNA under UV light. By comparing to known molecular weight standard, the size of the separated fragments was estimated.

**Table 12:** TAE Buffer (X50) pH~8.5 stock solution

| Product | Amount | Concentration (x50) |
|---|---|---|
| Tris-Base | 242gr | 2M |
| Glacial Acetic-Acid | 57.1ml | 2M |
| 0.5M EDTA (pH 8.0) | 100ml | 50mM |
| DDW | Adjust to 1000ml | |

**Table 13:** 1-2% agarose gel preparation

| Product | Amount | Comments |
|---|---|---|
| Agarose | 1-2% | Percent of Gel (%) $= \frac{\text{Agarose\_Weigh(gr)}}{\text{Volume}(ml)} \times 100$ |
| TAE | 60ml for small tray,<br>120ml for large tray | 90-105 sec in microwave |
| Et-Br | 1 drop for small tray,<br>2 drops for large tray | Pure in hood and allow to solidify for about 20 minutes |

### *6.3.2 DNA sequencing*

DNA sequencing for different reaction products were performed using the services of Macrogen Europe, Amsterdam the Netherlands (europe@macrogen.com).

### *6.3.3 Computer analysis of sequencing results*

DNA sequence analysis and alignment were preformed using the Benchling tool (Benchling, Inc.) and BLAST tool (National Institutes of Health)[59].

## 6.4 Heat shock transformation into DH5α / STbl2

Eppendorf tube containing 100µl of competent cells (CC) was defrosted on ice for 10-15 minutes, while a wet bath was pre-heated to 42ºC. Once the cells were thawed, 5µl of reaction products (ligation or Gibson) / purified DNA plasmid were added, and the cells were incubated with the DNA for 30 minutes on ice. During the incubation, SOC solution was prepared and pre-heated to 37˚C. Cells were then transferred to the pre-heated wet bath for 45 sec, and immediately transferred to ice for 2 min incubation. Followed by incubation, 900µl of pre-heated SOC were added and the culture was recovered for 1hr at 37 ˚C shaker incubator, 250 rpm in two-stage tube. After the recovery, the culture was transferred to 1.5ml Eppendorf tube and centrifuged for 30 sec at max speed. Then, the excess supernatant was discarded, leaving about 100µl, that were used to re-suspend the pellet followed by plating the culture on an agar plate containing an appropriate antibiotic. The plate was incubated O/N at 37˚C incubator.

### *6.4.1 Colony Isolation*

Colonies were picked from the transformation plate and gently spread on a new backup agar plate (containing an appropriate antibiotic) using a sterile tip, which then placed in a tube containing colony PCR reaction mix for first cloning verification. DNA was extracted from colonies that were suspected as positive according to colony PCR, digested using restriction enzymes for further verification, and then sent for sanger sequencing.

### *6.4.2 Integration verification by PCR*

To validate successful genomic integration into yeast cells after verification by fluorescence microscope, genomic DNA (gDNA) was extracted using Smash&Grab buffer. The extracted gDNA was used as a template for PCR with primers that were designed to amplify fragment containing both chromosomal and plasmid DNA. The amplified product was then cleaned by NucleoSpin Gel and PCR Clean-up kit and sent for sanger sequencing.

#### *6.4.2.1 Genomic DNA extraction using Smash & Grab buffer*

A single yeast colony was inoculated into 5ml YEPD for over-night incubation at 30˚C, 200rpm. 1.5ml of the culture was centrifuged for 1 min and 30 sec at ~16,000xg and the supernatant was discarded. the pellet was re-suspended in 200µl smash and grab buffer (1% SDS, 2% Triton X-100, 100mM Nacl, 100mM Tris pH 8, 1mM EDTA)[60]. Glass beads, 100µl phenol and 100µl chloroform were added (in the chemical hood), then the tubes were shaken in a bead beater homogenizer for 3min, centrifuged for 3 min (~16,000xg) and the clear supernatant was transferred into a new tube (in the chemical hood). RNaseA (1µl of 50mg/ml) was added and the tubes were incubated for 10min in the chemical hood. gDNA was precipitated using 3M Nacl (10% volume) and 100% Ethanol (200% volume), incubated on ice for at least 5min, then centrifuged for 2 min (~16,000Xg at 4°C) and the supernatant was discarded. Pellet was washed with 600ml 70% ethanol, re-centrifuged and re-suspend in 100ml filtered DDW/MBW.

### *6.4.3 Yeast cells fixation*

A single yeast colony was inoculated using a sterile loop into 5ml YPD medium for O/N incubation at 30˚C, 200 rpm (starter). The cell density of the starter was measured after about 18 hours, and the culture was diluted to $O.D_{600}$~0.3 with YPD to a final volume of 20ml in 50ml Erlenmeyer. The diluted culture was shaken at 30˚C 200rpm for ~150min until it reached the mid log phase ($O.D_{600}$ 0.5-0.9). Then, 450µl culture was transferred into a new Eppendorf tube containing 45µl 37% Formaldehyde and mixed by inversion. The mixture was then incubated for 15 min at room temperature and centrifuged for 5 min at 8000rpm. The supernatant was discarded, and the pellet was resuspended with 1ml sterilized 0.1M potassium phosphate. The resuspended pellet was centrifuged again for 1 min and 30 sec at 3500g, the supernatant was discarded, and the pellet resuspended in 100µl sterilized 0.1M potassium phosphate[61].

### *6.4.4 Yeast nucleus tagging by Hoechst*

1ml of Mid-log phase culture was transferred into a two-stage tube and 1µl of 4mg/µl Hoechst dye was added. The mixture was incubated for 15min in 30ºC at 200tpm prior microscope live cell visualization.

## 6.5 Yeast transformation

### *6.5.1 High efficiency LiAC/SS-DNA/PEG transformation*

A single yeast colony was inoculated using a sterile loop into 5ml YPD medium for O/N incubation at 30˚C, 200 rpm (starter). The cell density of the starter was measured after about 18 hours, and the culture was diluted to $O.D_{600}$~0.3 with YPD to a final volume of 20ml in 50ml Erlenmeyer. The diluted culture was shaken at 30˚C 200rpm for ~150min until it reached the mid-log phase ($O.D_{600}$ 0.5-0.9). Next, the culture transferred to 50ml falcon, centrifuged for 5 min at 3500g and the supernatant was discarded. The culture was transferred to a 1.5ml Eppendorf tube and washed twice using 1ml sterilized DDW followed by centrifugation for 1.5 min at 2500g. After two washing steps the pellet was re-suspended in 900µl sterilized DDW and 100µl sterilized 1M LiAc and the culture rotated in Jntelli-Mixer for 20-30min. Then, the culture was centrifuged for 1.5 min at 2500g, the supernatant was discarded, and the pellet was re-suspended in 150-200µl sterilized DDW. The cells were ready for transformation at this point.

Each transformation tube contained:

- 240µl 50% PEG 3350
- 36µl 1M LiAc
- 10µl ssDNA (salmon sperm DNA); Incubated for 10min in 95˚C and incubated on ice until use
- At least 1000ng DNA of interest (linearized at the homologous site for integration)
- 50µl competent yeast cells
- Adjust with sterilized DDW to a final volume of 360µl

Transformation tube was vortex and incubated in a pre-heated (42˚C) wet bath for 45-60min. Culture was centrifuged for 30sec at 2500g, the supernatant was discarded, the pellet was re-suspended in 100µl sterilized DDW and plated on SD agar plate missing the relevant amino acid marker. The plates were incubated at 30˚C for 2-3 days[62].

#### *6.5.1.1 CRISPR/Cas9 based transformation*

Transformation of linearized DNA using CRISPR/Cas9 is based on LiAC/SS-DNA/PEG transformation. gRNA for DNA integration at a specific locus was designed by ATUM tool (https://www.atum.bio/eCommerce/cas9/input) and ordered as a Forward and Reverse oligo from IDT, with the addition of overhangs compatible for BplI digestion at the 3’ end. The oligos were ligated and inserted into a bRA66 plasmid (Addgene #100952) that was digested with BplI restriction enzyme and cleaned from agarose gel. bRA66 plasmid also contains a gene encoding

the Cas9 protein under GAL1 promoter and a gene that provides resistance for yeast to the Hygromycin B antibiotic.

bRA66 plasmid after insertion of gRNA was integrated together with a linearized DNA that served as a templet for DNA double strand break by the Cas9 nuclease.

Each transformation tube contained:

- 240µl 50% PEG 3350
- 36µl 1M LiAc
- 10µl ssDNA (salmon sperm DNA); Incubated for 10min in 96˚C and incubated on ice until use
- At least 7500ng of bRA66+gRNA plasmid
- At least 3000ng of linearized dsDNA that serves as a donor and has homologous ends compatible to the sites generated after the cleavage of the Cas9 nuclease
- 50µl competent yeast cells
- Adjust with sterilized DDW to a final volume of 360µl

Transformation tube was vortex and incubated in pre-heated (42˚C) wet bath for 45-60min. Culture was centrifuged for 30sec at 2500g, the supernatant was discarded, the pellet was resuspended in 100µl sterilized DDW and plated on YPGal+Hygromycin (200µg/ml). The plates were incubated at 30˚C for 2-3 days

### *6.5.2 Colony Isolation*

Colonies were picked from the transformation plate and gently spread on a new agar plate (missing the relevant amino acid marker) using a sterile tip, which was then placed in a tube containing colony PCR reaction mix for first cloning verification. The colonies that were seemed to be positive according to the colony PCR results were checked for successful expression under the microscope (in case the insert included fluorescent protein).

## 6.6 Yeast strains construction

### *6.6.1 LexA repressor fused to iRFP720 fluorescent protein*

For the construction of the plasmid containing the gene encoding the LexA binding protein fused to iRFP720 fluorescent protein, iRFP720 sequence was amplified from pNLS-iRFP720 plasmid (Addgene #45467) by PCR using the oligos F/R_iRFP720_Insert_Gibson, and homologous regions compatible to FRP991_PACT1(-1-520)-LexA-ER-Gal4AD plasmid (Addgene #58438) were added at the ends of sequence by PCR using the oligos F/R_iRFP720_BB_Gibson, designed to replace ER-LBD gene. iRFP720 sequence was inserted by Gibson assembly reaction and the cloning was verified by sanger sequencing, resulting in plasmid pYS0046.

Next, the plasmid was integrated into the genome of W303-1B *saccharomyces cerevisiae* yeast strain (YA204) by first linearizing the plasmid with NheI-HF restriction enzyme, followed by high

efficiency yeast integration protocol as described in section 6.5.1, resulting in YS0032 yeast strain. Transformed cells were plated on synthetic defined (SD) plates without Histidine and incubated for 2 days at 30ºC incubator. Single colonies were picked and isolated on a new SD -HIS3 plate, then transferred to YPD plates. The colony with the highest fluorescence intensity as visualized under fluorescence microscope was selected.

### *6.6.2 Constructing LexA operator tandem repeats for single locus labeling*

LexA operator tandem repeats were generated starting with 8 LexA operator repeats plasmid (Addgene #58435). The construction of Lex binding sites array was done using three different restriction enzymes (XbaI, PacI and AvrII) and relied on compatible sticky ends generated by XbaI and AvrII, which after ligation generated a restriction site that cannot be recognized by either enzyme. The plasmid was digested once with PacI and AvrII and once with PacI and XbaI, followed by recovery of restricted products out of an agarose gel. Ligation, as shown in Figure 14, doubled the number of operator sites with each restriction-ligation cycle but did not alter the restriction site map of the plasmid. Three rounds of this cloning cycle generated approximately 50-65 repeat plasmid (several repeats were reduced during the cloning cycles), resulting in plasmid pYS0047. After three cycles the plasmid was no longer stable in the *E.Coli* strain DH5α.

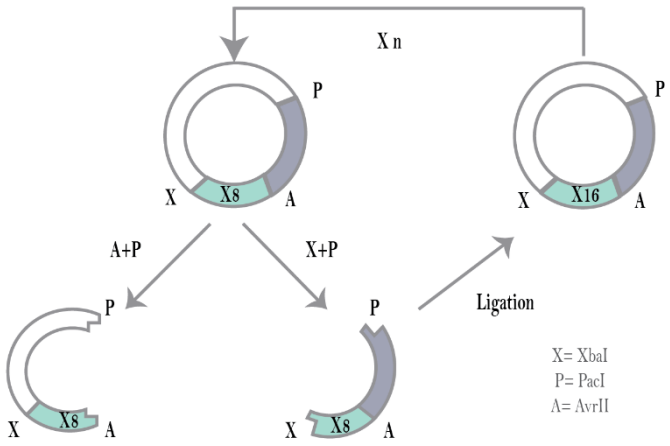



**Figure 14: Cloning strategy to generate multiple LexA operator repeats array**. using PacI, AvrII and XbaI enzymes, as diagramed above, doubled the LexA operator repeat number with each cloning cycle while the restriction map remained unchanged.

Next, the plasmid was integrated into the genome of YS0032 yeast strain by first linearized the plasmid with BstBI restriction enzyme, followed by high efficiency yeast integration protocol, resulting in YS0042 yeast strain. Transformed cells were plated on synthetic defined (SD) medium without Histidine and Uracil (SD -HIS3 -URA3) and incubated for 2 days at 30ºC incubator. Single colonies were picked and isolated on a new SD -HIS3 -URA3 plate, then transfered to YPD plates.

The colony with the brightest localized spot as visualized under fluorescence microscope was selected.

### *6.6.3 Constructing LexA repressor fused to iRFP720 fluorescent protein with the addition of SV40 NLS*

The fusion of nuclear localization signal (NLS) to the LexA repressor-iRFP720 fluorescent protein fused gene was done by opening the plasmid by PCR using the oligos F/R_BB_iRFP720_NLS_C without the LexA repressor-iRFP720 fused gene, and amplify this construct in another PCR reaction with oligos F/R_Insert_iRFP720_NLS_C which added the SV40 signal to the C-terminus end and homologous regions compatible to the open vector. The amplified product LexA-iRFP720-SV40 was inserted into the open vector by Gibson assembly reaction, and the cloning was verified by sanger sequencing.

The plasmid was integrated into the genome of YA204 yeast strain as described in section 6.6.1, resulting in YS0058 yeast strain. The colony with the highest fluorescence intensity in the nucleus as visualized under the microscope was selected. Next, pYS0047 plasmid was integrated as described in section 6.6.2, and the colony with the brightest localized spot was selected.

### *6.6.4 Constructing LexA operator tandem repeats for labeling two mating type switching related loci*

To label two loci involved in mating type switching event (HMRa and HMLα), a plasmid containing a gene encoding the fused protein LexA repressor-iRFP720-SV40 under Trp1 promoter instead of His3 promoter was constructed. The plasmid was opened by PCR using F/R_Trp1_BB_LexA_iRFP720 oligos to exclude the His3 marker, and Trp1 sequence was amplified from pYS0005 by PCR using F/R_Trp1_Insert_LexA_iRFP720 oligos that added homologous regions for the open plasmid. Trp1 sequence was inserted by Gibson assembly reaction and the plasmid was sent for sanger sequencing for verification. The plasmid was linearized by digestion using Bsu36I restriction enzyme or by PCR using F/R_Trp1_LexA_Open oligos, then integrated into the genome of YS0040 yeast strain, resulting in YS0060 yeast strain. The transformed cells were plated on a SD plate without His3, Ura3 and Trp1 and incubated for 2 days at a 30ºC incubator. Single colonies were picked and isolated on a new SD -HIS3 -URA3 -TRP1 plate, then transformed to YPD plates. The colony with the highest fluorescence intensity signal of iRFP720 all over the nucleus as visualized under a fluorescence microscope was selected.

To generate a plasmid containing 64 repeats of LexA operators with ADE2 marker (instead of URA3) that can be integrated at HMRa locus, a plasmid containing 32 LexA operator repeats (pYS0074) that was constructed as described above was digested with PfoI and PacI restriction enzymes and recovered from agarose gel. ADE2 gene was amplified from pIL01 plasmid by PCR using the oligos F_ADE2_LexAx64_PfoI and R_ADE2_LexAx64_PacI that added the PfoI and PacI restriction sites at the ends of the amplified product. The amplified product was digested with PfoI and PacI and inserted to the digested plasmid by ligation. The cloning was verified by sanger

sequencing. Homologous region for HMRa locus (ChrIII:295192-295337) was ordered as gBlock (IDT) with XmaI and PstI restriction sites at the edges. The plasmid containing 32 LexA operator repeats with ADE2 yeast marker and the gBlock were digested with XmaI and PstI-HF restriction enzymes, followed by recovery and purification from agarose gel, then the gBlock was inserted by ligation. The cloning was verified by sanger sequencing. The number of the operator repeats was doubled then by the strategy described above to generate approximately 20 operator repeats (most of the repeats were reduced during the process), resulting in plasmid pYS0093. The plasmid was linearized at the homologous region for HMR using pSPXI restriction enzyme, and transformed to YS0060 (yeast strain labeled with Tet system at HMLα) as described at 6.6.1 section, using SD -His3 -Ura3 -Trp1 -Ade2 plate, resulting in YS0062 yeast strain. The colony bare two bright localized spots at both HMRa and HMLα as visualized under the microscope was selected.

### *6.6.5 LacI repressor fused to iRFP720 fluorescent protein under His3 promoter*

A gene that encoding LacI protein was amplified from pKW3034 by PCR using the oligos F/R_LacI_iRFP720_Insert, which added homologous regions for the plasmid containing the fusion LexA repressor-iRFP720 gene. The plasmid was opened by PCR using the oligos F/R_Characterization_BB that excluded the LexA repressor gene, the LacI gene was inserted using Gibson assembly reaction and the cloning was verified by sending the plasmid for sanger sequencing (using F_Protein_iRFP720_Seq oligo), resulting in plasmid pYS0075. The plasmid was linearized by digestion using NheI-HF restriction enzyme and was integrated into YA204 yeast genome as described in 6.6.1 section, resulting in YS0043 yeast strain.

### *6.6.6 TetR repressor fused to iRFP720 fluorescent protein under His3 promoter*

A plasmid containing the gene encoding TetR protein fused to iRFP720 fluorescent protein was constructed using the same strategy as described in 6.6.5 section, but with the oligos F/R_TetR_iRFP720_Insert to amplify the TetR gene from pKW3034 plasmid, resulting in plasmid pYS0077. The plasmid was integrated as described in 6.6.5 section, resulting in YS0044 yeast strain.

### *6.6.7 Lambda cI repressor fused to iRFP720 fluorescent protein under His3 promoter*

A plasmid containing the gene encoding Lambda cI protein fused to iRFP720 fluorescent protein was constructed using the same strategy as described in 6.6.5 section, but with the oligos F/R_Lambda_iRFP720_Insert to amplify the Lambda cI gene from pIL01 plasmid, resulting in plasmid pYS0076. The plasmid wad integrated as described in 6.6.5 section, resulting in YS0045 yeast strain.

### *6.6.8 256 LacO repeats for integration into URA3 locus*

A plasmid containing 256 repeats of Lac operator under URA3 promoter instead of LEU2 was constructed by restriction digestion with PfoI and SacI-HF of both pSR6 plasmid containing 256 LacO repeats to exclude LEU2 marker and pYS0017 to generate URA3 sequence with overhangs

compatible for the two restriction enzymes. Both restricted products were recovered from agarose gel. URA3 sequence was inserted by ligation and the cloning was verified by sending the plasmid for sanger sequencing, resulting in plasmid pYS0079. The plasmid was linearized by digestion using BstBI restriction enzyme and integrated into YS0043 as described in 6.6.2 section, resulting in YS0048 yeast strain.

### *6.6.9 64 LambdaO repeats for integration into URA3 locus*

A plasmid containing 64 repeats of Lambda operator under URA3 promoter instead of HIS3 was constructed by restriction digestion with PfoI and NsiI-HF of both pIL02 plasmid containing 64 LambdaO repeats to exclude HIS3 marker and pYS0017 to generate URA3 sequence with overhangs compatible for the two restriction enzymes. Both restricted products were recovered from agarose gel. URA3 sequence was inserted by ligation and the cloning was verified by sending the plasmid for sanger sequencing, resulting in plasmid pYS0078. The plasmid was linearized by digestion using BstBI restriction enzyme and integrated into YS0045 as described in 6.6.2 section, resulting in YS0046 yeast strain.

## 6.7 3D Point-Spread-Function (PSF) Engineering optical setup

The experimental optical set up used in this research is similar to the one used in a previous study, utilizing PSF engineering[42]. The setup includes a Laser box (Toptica iChrome MLE 405/488/561/640) and a NIDAQ card (NI PCI-6723 32 ch Analog Output Board) connected to conventional (inverted) fluorescent microscope (Nikon ECLIPSE T*i*-E or Nikon ECLIPSE T*i*-2) with high NA objective (CFI Plan Apo lambda 100X Oil, NA 1.45, WD 0.13mm/ CFI SR HP Apo TIRF 100X oil, NA 1.49, WD 0.12mm/ SR HP Apo 100X oil, NA 1.35, WD 0.3mm), and equipped with a PRIOR Piezo stage. The emission path includes a multi-band pass dichroic mirror (Chroma; TRF89902–ET-405/488/561/640,), and instead of receiving image in the standard image plane, a *4f* optical system was added by extending the emission light path, with a dielectric phase mask. The phase mask was fabricated on a fused silica substrate through three iterations of photolithography, with Refractive Ion Etching (RIE) following each iteration. The design was fabricated for 580 nm wavelength with NA objective of 1.35, 100x magnification and 4 microns Z-range.

The final PSF encoding the 3D location of the emitter out of a single widefield 2D image using fluorescence microscope equipped with either SCMOS PHOTOMETRICS (Prime 95B) or EMCCD Andor (iXon Life 897) cameras located at the new image plane (forth focal plane of the *4f* system). All images obtained in exposure time of 50-100ms and collected using the *NIS-ELEMENTS AR AUTO RESEARCH SOFTWARE (5.02.02).*

### *6.7.1 3D localization using VIPR*

Three-dimensional localization of the LexA-iRFP720 localized fluorescent spots was performed by choosing a region-of-interest (ROI) around the imaged cell (size 41x41 pixels) and fitting the ROI with a sum of a PSF and an asymmetrical 2D gaussian, aimed to model the emission from

non-localized fluorescent proteins scattered around the cell. For PSF fitting, the effective pupil function of the optical system was experimentally retrieved by VIPR[63]. The retrieved pupil function was used for a PSF-generating function which corresponds to a position of an emitter to a measured light distribution across the sensor plane. Per ROI captured, the most likely parameters[63] to describe the measured pixel values were fitted, based on the Poisson statistical properties of light.

### *6.7.2 Sample preparation for microscopy visualization*

A single yeast colony was inoculated into 5ml YPD or SC-LFM (2% glucose) for O/N incubation in 30˚C 200rpm (starter). The cell density of the starter was measured after about 18 hours, and the culture was diluted to $O.D_{600}$~0.3 with YPD or SC-LFM (2% glucose) to a final volume of 5ml in a glass culture tube. The diluted culture was shaken at 30˚C 200rpm for ~150min until it reached the mid-log phase. Since YPD has a reddish color, the culture grown on this medium was washed twice as described earlier to reduce the medium background, then re-suspended in 0.5-1ml of sterilized DDW. SC-LFM (2% glucose) is a colorless culture medium hence no washing was necessary, and the culture was ready for microscopy observation once the cells reached the mid log phase.

### *6.7.3 Microscopy 1.5% agarose slides preparation*

Yeast cells were immobilized during microscopy visualization using 1.5% agarose slides (Table 14). Thus, visualization of single cell DNA dynamic was enabled by eliminating cell migration.

**Table 14:** Microscopy 1.5% agarose slides

| Product | Amount | Comments |
|---|---|---|
| Agarose | 1.5% | Equation 1: $\text{Percent of Gel}(\%) = \frac{\text{Agarose\_Weigh}(\text{gr})}{\text{Volume}(ml)}$ |
| DDW | 6-9ml | 15-20sec in microwave<br>The amount depends on number of slides |

Each $22x22_{mm}$ coverslip is loaded with 400µl gel and immediately covered with $22x22_{mm}$ coverslip on top.

### *6.7.4 Scanning colonies using Imaging Flow Cytometer (IFC)*

Yeast cells were culture 1-2 days before the experiment. 200µl of mid-log phase culture were loaded into an Eppendorf tube and were inserted to ***Amnis® ImageStream®X Mk II Imaging Flow Cytometer***. The cells were illuminated with 640nm laser with band pass emission filter of 702/85.

## 6.8 Mammalian cell culture and transfection

### *6.8.1 Cell thawing*

Cells are taken out from liquid nitrogen and defrost in a water bath pre-warmed to 37ºC for few minutes. Thawed cells move to class II biological cabinet and a pre-heat medium is added by pipetting gently up and down until the mixture is completely homogenous. For suspension cell lines, the culture transfer to a disposable polycarbonate Erlenmeyer flask and shake in a 37℃ incubator with 8% $CO_2$ at 135rpm. For adherent cells, the culture is centrifuged for 5 minutes at 100g, the supernatant is vacuumed, and the pellet is resuspended in 5ml pre-warmed medium by pipetting gently up and down until there are no more clumps. The culture transfer to a new culture plate and stir laterally in an "eight"-shape very slowly. The plate incubates in a 37℃ incubator with 5% $CO_2$. After 24 hours the cells are observed under the microscope and cell density is determined by counting.

### *6.8.2 Cell splitting*

Before splitting, the viability and the density of the culture should be determined. For adherent cells, the medium is vacuumed from the edge of the old plate by tilting it to one side. The culture is washed with room-temperature phosphate buffered saline (PBS) by covering the entire plate and then vacuumed. Cells are detached from the plate using trypsin and by shaking the plate gently, followed by 1 min incubation in a 37ºC incubator. Fresh pre-warmed medium is added by pipetting the cells until there are no more clumps, then a small volume of the culture transfer to a new plate (depending on the desired dilution) containing fresh culture medium, stir laterally in an "eight"-shape, and incubate in a 37℃ incubator with 5% $CO_2$. For cells in suspension, a small culture volume (depending on the desired dilution) is transfer to a fresh, pre-warmed medium in a new disposable polycarbonate Erlenmeyer flask to give a final cell density in the desired final volume. The flasks are shaken in a 37℃ incubator with 8% $CO_2$ at 135 rpm.

The culture is typically splitting every 48-72 hours, depending on the cell density, usually to a final density of $0.1 \times 10^6$-$0.2 \times 10^6$ viable cells/ml.

### *6.8.3 Cell freezing*

The cells should be cultured a few days before freezing to the desired density (usually 0.5 to 1 x $10^6$ viable cells/ml), then transfer to a sterile 15ml falcon. The viability and cell density are determined by counting and the volume of the freezing medium required to yield a final cell density of 10 x $10^6$ viable cells/ml is determined. Cells are centrifuged at 100 x g for 5 minutes at room temperature and the medium is carefully aspirate. Cells are resuspended in the pre-determined volume of freezing medium, then 1ml of the cell suspension is aliquot into cryovials. Cells are freeze in an automated or manual, controlled-rate freezing apparatus following standard procedures. For ideal cryopreservation, the freezing rate should be a decrease of 1°C per minute. The frozen vials are then transferred to liquid nitrogen for long-term storage.

### *6.8.4 Cell transfection*

#### *6.8.4.1 HEK293 adherent cells transfection using Lipofectamine3000*

Cells are seeded 18 to 24 hours prior to transfection to reach 70-90% confluency at the transfection day, according to table 15.

**Table 15:** Seeding density and cells at confluency require for cell transfection in different flasks

| Flask | Seeding density | Cells at confluency | PBS | Trypsin | Growth medium |
|---|---|---|---|---|---|
| T-75 flask | $2.1\times10^6$ | $8.4\times10^6$ | 5 | 5 | 8-15 |
| T-175 flask | $4.9\times10^6$ | $23.3\times10^6$ | 17 | 17 | 35-53 |

At the day of transfection, the medium is vacuumed, and a fresh, pre-warmed medium is added at the same volume. Then, Lipofectamine/Opti-MEM mix is prepared in two different concentrations in two different tubes, according to table 16, and mixed well for 2-3 sec.

**Table 16:** OptiMEM/Lipofectamine3000 reagent require for different flasks

| Material | T-75 flask | T-175 flask |
|---|---|---|
| OptiMEM medium | 975x2 µl | 2.3x2 ml |
| Lipofectamine 3000 reagent | 29.3 and 58.5 µl | 69.8 and 139.5 µl |

A master mix of DNA diluted in Opti-MEM medium and P3000 reagent is prepared according to table 17 and mixed well.

**Table 17:** Diluted DNA/P3000 reagent require for different flasks

| Material | T-75 flask | T-175 flask |
|---|---|---|
| OptiMEM medium | 2 ml | 4.7 ml |
| DNA | 39 µg | 93 µg |
| P3000 reagent (2µl/µg DNA) | 78 µl | 186 µl |

Diluted DNA is then added to each tube of diluted Lipofectamine 3000 reagent (1:1 ratio), according to table 18. The mixture was incubated for 5 min at room temperature and added to the cells by dropping. The transfected cells were incubated at 37ºC incubator with 5% $CO_2$ for 2-3 days.

**Table 18:** Diluted DNA/diluted Lipofectamine3000 reagent require for different flasks

| Material | T-75 flask [µl] | T-175 flask [µl] |
|---|---|---|
| Diluted DNA (with P3000 Reagent) | 975 | 2325 |
| Diluted Lipofectamine3000 Reagent | ~1000 | ~2500 |

#### *6.8.4.2 HEK293-F FreeStyle cells transfection using branched Polyethyleneimine (PEI)*

HEK293-F FreeStyle cells were seed 24 hours before transfection at 0.7 x $10^6$ cells/ml into a final volume of 30 ml of Pre-warmed FreeStyle 293 expression medium in 125ml Erlenmeyer flask, to reach a cell density of 1.0 x $10^6$ cells/ml. On the day of transfection, the cells were count and their viability was determined. Then, 40 µg of filter-sterilized DNA (1 µg of DNA per million transfected cells) were added to 8 ml of PSB and the mixture was mixed vigorously for 3 sec. The mixture was incubated at room temperature for 20 min and then added to the cells by dropping. The transfected cells were incubated in an orbital shaker incubator for a further 48 hr at 37 °C, 135 rpm and 8% $CO_2$.

## 6.9 Protein purification and validation

### *6.9.1 Protein purification using His-tag column*

At least 48 hours post-transfection, the cell culture (in case of cells in suspension) or the supernatant (in case of adherent cells) were collected into a new 50ml falcon in a class II biological cabinet. The cells or supernatant were centrifuged at 4000g for 20 min at 4ºC using aerosol-tight caps, and the supernatant was transferred into a new 50ml falcon tube. The supernatant was filtered using a 0.22um Stericap filter and placed on ice until use. 600µl of Ni-NTA resin per 20ml culture were washed with double amount of PBS (1.2ml) in a 2ml Eppendorf tube and were centrifuged at 2000g for 10 min, then the PBS was discarded. The resin was resuspended with 1ml filtered supernatant, transferred into the falcon containing the rest of the supernatant and inverted 3 times. The resin was incubated with the supernatant for 2 hours on a roller shaker (Intelli mixer) at 10rpm (C1 program) at room temperature. A clean polypropylene column was loaded with the supernatant-resin mixture and the flow-through was collected in a 14ml falcon and placed on ice. The column was then washed with 10 column volume of wash buffer, and the wash solution was collected into a 14ml falcon and placed on ice. The bound protein was then eluted with 5 column volume of elution buffer into 5 SafeSealed microcentrifuge tubes for each fraction. All fractions were frozen in liquid nitrogen and then placed in a -80ºC freezer for long-term storage.

### *6.9.2 SDS polyacrylamide gel electrophoresis*

Casting stand and glass cassette were assembled according to the manufacturer instructions (Mini-PROTEAN tetra-cell, Bio-Rad) and tested for leakage with water. The separating gel was prepared by mixing the different solutions at a desired concentration according to Table 31 in a 50ml falcon tube.

**Table 19**: Preparation of separating gel in different concentrations

| %Acrylamide | 7% | 10% | 12% | 15% |
|---|---|---|---|---|
| Buffer Tris pH 8.8 | 4.95 ml | 4.95 ml | 4.95 ml | 4.95 ml |
| Glycerol 50% | 440 µl | 400 µl | 400µl | 400 µl |
| Acryl amid 40% | 3.5 ml | 5 ml | 6 ml | 7.5 ml |
| Distilled water | 11.15 ml | 9.65 | 8.5 ml | 7.15 ml |

Total volume = 20 ml

15µl of TEMED that served as catalyst and 100µl of APS that served as cross linker were added to the mixture. The tube was quickly inverted several times and the solution was filled in the space between the glasses using a 5ml pipettor, leaving about 2 cm from the top for the stacking gel. Water was then added on top of the gel to ensure the formation of a straight line. The excess gel was left in the falcon to verify solidification. Once the gel solidified, the water was removed and a stacking gel (4%) was prepared by mixing 4.5ml $dH_2O$, 1.75ml Tris(HCl)-SDS pH 6.8, 0.695ml 40% Acrylamide, 8µl TEMED and 40µl APS. The remaining space was filled with the stacking gel on top of the separating gel while avoiding air bubbles formation. Then, a comb with a desired number of wells was inserted and the gel was left for solidification. The gel is then wrapped with a wet paper and kept at 4ºC or immediately used.

If required, the samples were diluted in dH2O. Then, sample buffer x4 was added to the samples and boiled at 96ºC for 10 min. The cell electrophoresis module was assembled according to the manufacturer's instructions and the inner chamber was filled with running buffer x1 while the tank was filled halfway. Samples and protein marker were loaded at each well, the tank lid was closed in the correct orientation and was connected to the power supply. The parameters were set to 150 V for one gel and 200 V for two gels for approximately 30 min. Then, the gel cassette was removed from the tank and the glasses were gently separated using a small spatula. The stacking gel was removed, and the separating gel was placed in a small container filled with a staining solution. The gel was shaken with the staining solution for 30 min. The staining solution was discarded and the destaining solution was added and shake until bands have become visible.

# 7 Materials

## 7.1 Chemicals

Table 20 lists the chemicals used in this research

**Table 20:** Chemicals

| Chemical | Cat # | Manufacturer |
|---|---|---|
| Ethidium Bromide (Et-Br) | 280344 | Hy-labs |
| Ethanol ABS (EtOH) | 64-17-5 | Gadot group |
| Acetic-acid glacial | 401391 | Carlo Erba reagents |
| Agarose | 50004 | Lonza, Rockland, ME USA |
| Glass beads | BBI-8541701 | Sartorius |
| $MgSO_4$ | M1063 | SPECTRUM chemicals MFG Corp. |
| Tris (hydroxymethyl)-aminomethane | S1519 | |
| Glycine | GL157 | |
| Lithium acetate dehydrate (LiAc) | A17921 | Alfa Aesar (Thermo Fisher Scientific) |
| $MgCl_2$ | 12315 | |
| N'-Tetramethyl-ethylenediamine (TEMED) | A12536 | |
| Hydrochloric acid (HCL) 32% | 7647-01-0 | Bio-Lab ltd. Hayetzira Jerusalem Israel |
| Sodium chloride (NaCl) | 001903059100 | |
| Sodium hydroxide pearls (NaOH) | 001908059100 | |
| Chloroform | 000308052100 | |
| Biophenol saturated | 001691234400 | |
| Glycerol anhydrous | 000712050100 | |
| Ammonium Sulfate ($(NH_4)_2SO_4$) | 7783-20-2 | Merck KGaA, 64271 Darmstadt Germany |
| Potassium chloride (KCl) | 7447-40-7 | |
| $NaH_2PO_4$. $H_2O$ | 10049-21-5 | |
| Potassium phosphate ($KH_2PO_4$) | 04243-500G | Sigma-Aldrich |
| Ethylenediaminetetraacetic acid tetrasodium salt dihydrate (EDTA) | ED4SS-500G | |
| Poly(ethylene glycol) (PEG) 3350 | P3640-2KG | |
| Triton X-100 | X100-100ML | |
| Sodium dodecyl sulfate (SDS) | L5750-1KG | |
| Formaldehyde solution | F1635-500ML | |
| Polyvinyl alcohol (PVA) | 348406-25G | |
| bisBenzimide H 33342 trihydrochloride (Hoechst) | B2261-25MG | |
| Ammonium persulphate (APS) | A3678-25G | |
| β-mercaptoethanol | M6250-10ML | |

**Table 20:** Chemicals- continue

| Chemical | Cat # | Manufacturer |
|---|---|---|
| Acrylamide/bis-acrylamide, 40% | A7802-100ML | Sigma-Aldrich |
| Phosphate buffered saline (PBS) | P5368-10PAK | |
| Polyethyleneimine (PEI), 25kDa, Branched | 408727-100ML | |
| High Precision Microscope Cover Glasses 22 x 22 mm, Thickness No. 1.5H (0.170mm ± 0.005mm) | 0107052 | Marienfeld SUPERIOR (Germany) |
| Coomassie brilliant blue | 161-0406-10g | Bio-Rad |
| Trypan blue | TRB-100ML | Tivan Biotech |

Table 21 lists the chemicals used in this research for the preparation of growth media used for bacteria and yeast culture.

**Table 21:** Growth media chemicals

| Chemical | Cat # | Manufacturer |
|---|---|---|
| Bacto™ Yeast Extract | 212750 | BD (Difco Laboratories; Becton, Dickenson, and company) |
| Bacto™ Peptone | 211677 | |
| Bacto™ Tryptone | 211705 | |
| Bacto™ Agar | 214010 | |
| YNB w/o AA w/o Sulfate | CYN0501 | FORMEDIUM |
| YNB w/o AA w/o Sulfate w/o folic acid w/o riboflavin | CYN6501 | |
| D-(+)-Glucose | G5767-500G | Sigma-Aldrich |
| D-(+)-Galactose | 150615000 | ACROS |
| **Amino acids** | | |
| Adenine | A14906 | Alfa Aesar (Thermo Fisher Scientific) |
| L-Histidine | A10413 | |
| **Chemical** | **Cat #** | **Manufacturer** |
| L-Leucine | A12311 | Alfa Aesar (Thermo Fisher Scientific) |
| L-Tryptophan | A10230 | |
| L-Lysine | 1.05700.0100 | Merck KGaA, 64271 Darmstadt Germany |
| L-Tyrosine | 1.08371.0100 | |
| L-Methionine | 64319-25G-F | Sigma-Aldrich |
| Arginine | 11039-100G | |
| Uracil | 157300250 | ACROS |
| L-Glutamine | 03-020-1B | Biological Industries |
| **Antibiotics** | | |
| Ampicillin | Art.-Nr.K029.1 | ROTH (A2S) |

**Table 21:** Growth media chemicals- continue

| Chemical | Cat # | Manufacturer |
|---|---|---|
| Chloramphenicol | C0378-5G | Sigma-Aldrich |
| Hygromycin B | HYG1000 | Tivan Biotech |
| 1% Pen-Strep | 03-031-1B | Biological Industries |

## 7.2 Molecular biology reagents

Table 22 and Table 23 list the reagents and ready mixes used in this research for molecular biology reactions.

**Table 22:** Reagents list

| Reagent | Cat# | Manufacturer |
|---|---|---|
| UPW | 01-866-1B | BI - Biological Industries |
| AccuGENE™ MBW | 51200 | Lonza |
| $MgCl_2$ | B0510A | New England Biolabs (NEB) |
| DMSO | B0515A | |
| dNTPs | ATPs – N0440S<br>GTPs – N0442S<br>TTPs – N0443S<br>CTPs – N0441S | |
| Q5 reaction buffer (5x) | B9027S | |
| Q5 high GC enhancer (5x) | B9028A | |
| Gel loading dye – Purple (6x) | B7024S | |
| T4 DNA Ligase Buffer (10x) | B0202S | |
| Restriction buffers | CutSmart<br>NEB 1.1<br>NEB 2.1<br>NEB 3.1 | |
| Deoxyribonucleic acid, single stranded from salmon testes (ssDNA) | D7656-1ML | Sigma-Aldrich |
| Tango buffer (10x) | BY5 | ThermoFisher Scientific |

**Table 23:** Master Mix list

| Master Mix | Cat# | Manufacturer |
|---|---|---|
| Q5® High-Fidelity Master Mix (2X) | M0492 | New England Biolabs (NEB) |
| Gibson Assembly Master Mix (2X) | M5510A | |
| Taq Ready Mix (2X) | EZ-3006 | Hy labs |
| Phire Plant Direct PCR Mix (2X) | F-160S | ThermoFisher Scientific |

Table 24 lists the commercial kits used in this research. PCR, digested and gel products were cleaned using NucleoSpin **Gel and PCR Clean-up**. Plasmid purification was done using **NucleoSpin Plasmid EasyPure** mini or midi-prep. Purified DNA concentration from both kits was measured using a Spectrophotometer (Table 25), by measuring the absorbance at 260nm.

**Table 24:** Commercial kits list

| Kit | Cat# | Manufacturer |
|---|---|---|
| NucleoSpin Plasmid EasyPure | 740727.250 | MACHEREY-NAGEL |
| NucleoSpin Gel and PCR Clean-up, Mini kit | 740609.250 | |
| NucleoSpin Gel and PCR Clean-up, Midi kit | 740986.20 | |

**Table 25:** Spectrophotometer devices

| Device | Model | Manufacturer |
|---|---|---|
| NanoDrop 2000/2000c Spectrophotometer | ND-2000 | ThermoFisher Scientific |
| NP80 NanoPhotometer | NP80 | IMPLEN |

Table 26 lists the enzymes used in this research.

**Table 26:** Enzyme list

| Name | Cat# | Manufacturer |
|---|---|---|
| Restriction Enzymes | Different Cat#-s' | New England Biolabs (NEB) |
| Phusion Hot Start II DNA Polymerase | M0535L | |
| T4 DNA Ligase | M0202 | |
| **Name** | **Cat#** | **Manufacturer** |
| Q5 High-Fidelity DNA Polymerase | M0491 | New England Biolabs (NEB) |
| Taq DNA Ligase | M0208 | |
| Phusion High-Fidelity DNA Polymerase | M0530 | |
| PrimeSTAR HS DNA Polymerase | R010B | TaKaRa |
| RNaseA | EN0531 | ThermoFisher Scientific |
| Trypsin EDTA Solution A | 03-050-1A | Biological Industries |

## 7.3 Growth media and microbiology solutions

### *7.3.1 YEPD (YPD) medium*

| | |
|---|---|
| Yeast Extract | 10gr/lit |
| Peptone | 20gr/lit |
| Glucose | 20gr/lit |
| For agar plates | 1.5% agar was added |

Sterilization method: autoclave

### *7.3.2 SC medium*

| | |
|---|---|
| YNB w/o AA w/o Sulfate | 6.7gr/lit |
| Ammonium Sulfate | 5gr/lit |
| Glucose | 20gr/lit |
| Adenine | 40mg/lit |
| Histidine | 20mg/lit |
| Leucine | 80mg/lit |
| Lysine | 50mg/lit |
| Methionine | 40mg/lit |
| Tryptophan | 50mg/lit |
| Tyrosine | 30mg/lit |
| Uracil | 20mg/lit |
| Arginine | 20mg/lit |
| For agar plates | 1.5% agar was added |

For SD (LFM) the appropriate amino acid/s was discarded, when the cells had the gene integrated as a marker.

Sterilization method: 0.22μm membrane filter

### *7.3.3 SC (low fluorescent medium (LFM))*

| | |
|---|---|
| YNB w/o AA w/o Sulfate w/o folic acid w/o riboflavin | 6.9gr/lit |
| Ammonium Sulfate | 5gr/lit |
| Glucose / Galactose / Raffinose | 20gr/lit |
| Adenine | 40mg/lit |
| Histidine | 20mg/lit |
| Leucine | 80mg/lit |
| Lysine | 50mg/lit |
| Methionine | 40mg/lit |
| Tryptophan | 50mg/lit |
| Tyrosine | 30mg/lit |
| Uracil | 20mg/lit |
| Arginine | 20mg/lit |
| For agar plates | 1.5% agar was added |

For SD (LFM) the appropriate amino acid/s was discarded, when the cells had the gene integrated as a marker.

Sterilization method: 0.22μm membrane filter

### *7.3.4 LB medium*

| | |
|---|---|
| Yeast Extract | 5gr/lit |
| Tryptone | 10gr/lit |
| NaCl | 10gr/lit |
| For agar plates | 1.5% agar was added |

Following medium sterilization, ampicillin/chloramphenicol was added to a final concentration of 100µg/ml / 25 µg/ml, respectively.

Sterilization method: autoclave

### *7.3.5 SOB medium*

| | |
|---|---|
| Yeast Extract | 5gr/lit |
| Tryptone | 20gr/lit |
| NaCl | 0.58gr/lit |
| KCl | 0.19gr/lit |
| 1M $MgSO_4$ | 10ml/lit add after autoclave (final concentration: 10mM) |
| 1M $MgCl_2$ | 10ml/lit add after autoclave (final concentration: 10mM) |

For SOC add 2% Glucose 1M prior usage.

Sterilization method: autoclave

### *7.3.6 Antibiotic stock solution*

**Ampicillin (100 mg/mL dissolved in MBW)**

| | |
|---|---|
| Ampicillin powder | 2gr |
| MBW | 20ml |

Sterilization by filtration with 0.22µm membrane, storage in -20˚C

**Chloramphenicol (25 mg/mL dissolved in EtOH)**

| | |
|---|---|
| Chloramphenicol powder | 0.5gr |
| EtOH | 20ml |

Sterilization by filtration with 0.22µm membrane, storage in -20˚C

**Hygromycin B (50mg/mL dissolved in MBW)**

| | |
|---|---|
| Hygromycin B powder | 0.1gr |
| MBW | 20ml |

Sterilization by filtration with 0.22µm membrane, storage in -20˚C

### *7.3.7 Growth media for cell culture*

Table 27 lists the growth media used in this research for mammalian cell culture

**Table 27:** cell culture media list

| Name | Cat# | Manufacturer |
|---|---|---|
| Dulbecco's Modified Eagle Medium (DMEM) | 01-170-1A | Biological Industries |
| Fetal Bovine Albumin (FBS) | 04-007-1A | |
| Opti-MEM I Reduced Serum Medium | 31985062 | ThermoFisher Scientific |
| FreeStyle 293 Expression Medium | 12338018 | |

### *7.3.8 Glycerol stock medium for -80°C deep freeze*

**Bacterial strains**

Glycerol stock medium is made of 60% glycerol and 40% LB solution and sterilized by autoclave. Bacterial glycerol stock was prepared by mixing 500µl of O/N culture with 500µl of glycerol solution.

**Yeast strains**

Glycerol stock medium is made of 30% glycerol and 60% double-distilled water and sterilized by filtration with 0.22µm membrane. Yeast glycerol stock was prepared by mixing 500µl of O/N culture with 500µl of glycerol solution.

### *7.3.9 Gibson assembly master mix solution*

Table 28 and Table 29 list the home-made protocol based on the Gibson enzymatic assembly (one-step thermocycler DNA assembly)[58].

**Table 28:** Gibson assembly isothermal (ISO) reaction buffer (5X)

| Product Name | Amount |
|---|---|
| 1M Tris-HCl pH~7.5 | 3ml |
| 2M MgCl2 | 150µl |
| PEG 8000 | 1.5gr |
| Nicotinamide adenine dinucleotide (NAD+) 100mM | 300µl |
| 25mM dNTP (from each: A, T, G, C)<br>Total 100mM dNTP | 60µl<br>Total: 240µl |
| 1M Dithiothreitol (DTT) | 300µl |
| MBW | Adjust to final volume of 6ml (~2.01ml) |

Sterilization by filtration with 0.22µm membrane, storage in -20˚C.

**Table 29:** Gibson assembly master mix

| Product Name | Amount |
|---|---|
| isothermal (ISO) reaction buffer (5X) | 320µl |
| 10 U/µl T5 Exonuclease | 0.64µl |
| 2 U/µl Phusion High-Fidelity DNA Polymerase | 20 |
| 40 U/µl Taq ligase | 160µl |
| MBW | Adjust to final volume of 1.2ml (~0.7ml) |

Aliquot to 15µl and keep in -20˚C.

## 7.4 Solutions and buffers for protein purification

### *7.4.1 Buffers for proteins purification using Ni-NTA resin column*

**Wash buffer (1 Liter)**

$NaH_2PO_4$. $H_2O$ 7.94 gr

NaCl 17.54 gr

Imidazole 1.36 gr (final concentration- 20 mM)

DDW 1 L

**Elution buffer (1 Liter)**

$NaH_2PO_4$. $H_2O$ 7.94 gr

NaCl 17.54 gr

Imidazole 16 gr (final concentration- 235 mM)

DDW 1 L

The solutions were filtered using 0.22um Stericup vacuum filtration system

### *7.4.2 SDS polyacrylamide gel electrophoresis solutions and buffers*

**Destain solution**

200 ml Absolute ethanol (or Methanol)

100 ml Acetic acid

700 ml $ddH_2O$

**Stain solution**

2.5 gr Coomassie brilliant blue

500 ml Ethanol

100 ml Acetic acid glacial

400 ml $ddH_2O$

**0.5 M Tris(HCl)-SDS pH 6.8**

6.05 gr Tris

0.4 gr SDS (or 4 ml of 10% SDS solution)

Adjust pH to 6.8

$ddH_2O$ up to 100 ml

**1.5 M Tris(HCl)-SDS pH 8.8**

45.5 gr Tris

1 gr SDS (or 10 ml 10% SDS solution)

Adjust pH to 8.8

$ddH_2O$ up to 250 ml

**Sample buffer x4**

1 ml 0.5 M Tris(HCl)-SDS pH 6.8

1.6 ml 10% SDS solution

0.8 ml glycerol

0.4 ml β-mercaptoethanol

0.1 ml 4% Bromophenol blue

4 ml $ddH_2O$

**Running buffer x10**

31 gr Tris

144 gr Glycine

10 gr SDS

$ddH_2O$ up to 1000 ml

## 7.5 DNA and protein ladders

Table 30 lists the DNA and protein ladders used in this research.

**Table 30:** DNA and protein ladders

| Name | Cat# | Manufacturer |
|---|---|---|
| ExcelBand 100bp DNA Ladder | DM2100 | SMOBIO |
| ExcelBand 1Kb Plus DNA Ladder | DM3200 | |
| PageRuler Prestained Protein Ladder | 26617 | ThermoFisher Scientific |

## 7.6 Plasmids

Table 31 lists the plasmids that were created or used in this research. For each plasmid specific details are provided regarding the source, antibiotic resistance, and function.

**Table 31:** Plasmids list

| Plasmid name | Source | Resistance | Description |
|---|---|---|---|
| pSR6 | Prof. Bystricky lab<br>Lassadi et al. 2015 | Ampicillin | 256 repeats of LacO binding sites<br>Marker for yeast integration: HIS3<br>(Linearization by NheI) |
| pKW3034 | Prof. Weis lab<br>Dultz, E. et al. 2018 | Ampicillin | HISp: LacI-eGFP; URAp: TetR-3*mCherry;<br>Marker for yeast integration: HIS3<br>(Linearization by NheI) |
| pKW2837 | Prof. Weis lab<br>Dultz, E. et al. 2016 | Ampicillin | 112 TetO repeats for integration in YBR022 Chr II (POA CDS)<br>Marker for yeast integration: URA3<br>(Linearization by BlpI / MluI) |
| pIL01 | Prof. Bystricky lab<br>Lassadi et al. 2015 | Ampicillin | HISp: LacI-CFP; URAp:λI-YFP; ADH1t;<br>Marker for yeast integration: ADE2<br>(Linearization by StuI at ADE2) |
| pIL02 | Prof. Bystricky lab<br>Lassadi et al. 2015 | Ampicillin | 64 λ-O repeats<br>Marker for yeast integration: HIS3<br>(Linearization by AscI in the CaURA3 flanking region) |
| pNLS-iRFP720 | Addgene #45467 | Kanamycin | iRFP720 fluorescent protein gene fused to SV40 nuclear localization signal for mammalian expression |
| FRP991_PACT1(-1-520)-LexA-ER-Gal4AD | Addgene #58438 | Ampicillin | LexA-ER-Gal4AD fusion gene<br>Marker for yeast integration: HIS3<br>(Linearization by NheI-HF) |

**Table 31:** Plasmid list- continue

| Plasmid name | Source | Resistance | Description |
|---|---|---|---|
| pYS0005 | Prof. Bystricky lab | Ampicillin | 256 repeats of LacO binding sites<br>Marker for yeast integration: Trp1<br>(Linearization by Bsu36I) |
| pYS0017 | Addgene #58435 | Ampicillin | 8 LexA operator repeats<br>Marker for yeast integration: URA3<br>(Linearization by BstBI at URA3) |
| pYS0019 | Prof. Shechtman lab | Ampicillin | 112 TetO reprats for integration in HML region - Chr III<br>Marker for yeast integration: URA3<br>(Linearization by BmgBI at HML region) |
| pYS0021 | Prof. Shechtman lab | Ampicillin | 256 LacO repeats for integration at Chr III MAT region<br>Marker for yeast integration: LEU2<br>(Linearization by PmlI in the MAT region) |
| pYS0046 | This research | Ampicillin | LexA binding protein fused to iRFP720 fluorescent protein (LexA binding protein sequence was taken from Addgene#58438).<br>Marker for yeast integration: HIS3<br>(Linearization by NheI) |
| pYS0047 | This research | Ampicillin | 64 repeats of LexA binding sites (was prepared from Addgene #58435-LexAx8)<br>Marker for yeast integration: URA3<br>(Linearization by BstBI) |
| pYS0074 | This research | Ampicillin | 32 repeats of LexA binding sites (was prepared from Addgene #58435-LexAx8)<br>Marker for yeast integration: URA3<br>(Linearization by BstBI) |
| pYS0075 | This research | Ampicillin | LacI binding protein fused to iRFP720 fluorescent protein<br>Marker for yeast integration: HIS3<br>(Linearization by NheI) |
| pYS0076 | This research | Ampicillin | λcI binding protein fused to iRFP720 fluorescent protein<br>Marker for yeast integration: HIS3<br>(Linearization by NheI) |

| | | | |
|---|---|---|---|
| pYS0077 | This research | Ampicillin | TetR binding protein fused to iRFP720 fluorescent protein<br>Marker for yeast integration: HIS3 (Linearization by NheI) |

**Table 31:** Plasmids list-continue

| Plasmid name | Source | Resistance | Description |
|---|---|---|---|
| pYS0078 | This research | Ampicillin | 64 repeats of LambdaO binding sites<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| pYS0079 | This research | Ampicillin | 256 repeats of LacO binding sites (made from pSR6)<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| pYS0082 | Prof. Arava Lab<br>Levi, O. et al, 2019 | Bacteria-Ampicillin<br>Yeast-Hygromycin | Cas9 expression under Gal promoter. gRNA scaffold for gRNA insertion using BplI restriction enzymes (Addgene #100952) |
| pYS0083 | Prof. Shechtman lab | Bacteria-Ampicillin<br>Yeast-Hygromycin | Cas9 expression under Gal promoter. gRNA for Chr.XV (724991-725010) |
| pYS0093 | This research | Ampicillin | Approximately 20 repeats of LexA binding sites and homologous region for HMR (Chr.III 295192-295337)<br>Marker for yeast integration: ADE3 (linearization by AflII) |
| pYS0094 | This research | Ampicillin | LexA binding protein fused to iRFP720<br>Marker for yeast integration: Trp1 (linearization by Bsu36I) |
| pYS0095 | This research | Ampicillin | LexA binding protein fused to iRFP720 with NLS at C-term<br>Marker for yeast integration: Trp1 (linearization by Bsu36I) |
| pYS0096 | This research | Ampicillin | LexA binding protein fused to iRFP720 with NLS at C-term<br>Marker for yeast integration: His3 (linearization by NheI) |

## 7.7 Bacterial strains

1. Bacterial strain *Escherichia coli* (DH5α) was kindly provided by Prof. Oded Beja lab.
   Genotype: $F^-$ ϕ80*lac*ZΔM15 Δ(*lac*ZYA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17($r_K^-$, $m_K^+$) *pho*A *sup*E44 $\lambda^-$ *thi*-1 *gyr*A96 *rel*A1
2. Bacterial strain *Escherichia coli* (STbl2) was purchased from ThermoFisher Scientific
   Genotype: $F^-$ *mcr*A Δ(*mcr*BC-*hsd*RMS-*mrr*) *rec*A1 *end*A1*lon gyr*A96 *thi sup*E44 *rel*A1 $\lambda^-$ Δ(*lac-pro*AB)

## 7.8 Mammalian cells

1. HEK 293 adherent cells

DNA profile: Amelogenin: X, CSF1PO: 11,12, D13S317: 12,14, D16S539: 9,13, D5S818: 8,9, D7S820: 11,12, TH01: 7,9.3, TPOX: 11, vWA: 16,19

## 7.9 Yeast strains

Table 32 lists the yeast strains that were used or created in this research. For each strain specific details are provided regarding the source, genotype, and function.

**Table 32:** Strains list

| Strain name | Origin/ Mother strain | Source | Genotype | Description |
|---|---|---|---|---|
| BY4741 | S288C | Prof. Arava lab | MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 | Brachmann CB et al, 1997 |
| BY4742 | S288C | Prof. Arava lab | MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Brachmann CB et al, 1997 |
| YA619 | W303-1B | Prof. Arava lab | MATa, ade2-1, can1-100, his3-11, leu2-3, trp1-1, ura3-1 | Tisi R et al, 2008 |
| YA204 | W303-1A | Prof. Arava lab | MATα leu2 his3 ade2 trpl ura3 | Navarre C et al, 1994 |
| YS0013 | BY4741 | Prof. Shechtman lab | MATa his3Δ1::pKW3034 leu2Δ0 met15Δ0 ura3Δ0 HML:: pYS0019 MAT:: pYS0021 | Chr III MAT region (197,202-197,392bp):: pYS0021 LacI-eGFP localization (256*LacO repeats) Chr III HML region (15088-15621bp):: pYS0019 TetR-mCherry localization (112*TetO repeats) Chr XV his3Δ1:: pKW3034 (LacI-eGFP & TetR-3mCherry) Marker for yeast integration: LEU2 (Linearization by PmlI) |

**Table 32:** Strains list-continue

| Strain name | Origin/ Mother strain | Source | Genotype | Description |
|---|---|---|---|---|
| YS0015 | YA619 | Prof. Shechtman lab | MATa, ade2-1, can1-100, his3-11::pKW3034 (HIS3), leu2-3, trp1-1, ura3-1 | Fused Fluorescent Repressors spread all over the nucleus Chr XV his3Δ1:: pKW3034 (LacI-eGFP & TetR-3*mCherry ) Marker for yeast integration: HIS3 (Linearization by NheI-HF) |
| YS0016 | BY4741 | Prof. Shechtman lab | MATa his3Δ1::pYS0046 leu2Δ0 met15Δ0 ura3Δ0 | $LexA_{bp}$ repressor fused to iRFP720 fluorescent protein Fluorescence all over the nucleus Chr XV his3Δ1:: pYS0046 ($LexA_{bp}$-iRFP720) Marker for yeast integration: HIS3 (Lineatization by NheI-HF) |
| YS0017 | BY4742 | Prof. Shechtman lab | MATα his3Δ1::pYS0046 leu2Δ0 lys2Δ0 ura3Δ0 | $LexA_{bp}$ repressor fused to iRFP720 fluorescent protein Fluorescence all over the nucleus Chr XV his3Δ1:: pYS0046 ($LexA_{bp}$-iRFP720) Marker for yeast integration: HIS3 (Lineatization by NheI-HF) |
| YS0032 | YA204 | This research | MATα leu2 his3::pYS0046 ade2 trpl ura3 | $LexA_{bp}$ repressor fused to iRFP720 fluorescent protein Fluorescence all over the nucleus Chr XV his3:: pYS0046 (LexA-iRFP720) Marker for yeast integration: HIS3 (Linearization by NheI-HF) |
| YS0035 | YS0032 | This research | MATα leu2 his3::pYS0046 ade2 trpl ura3::pYS0074 | $LexA_{bp}$ repressor fused to iRFP720 fluorescent protein& 32 repeats of $LexA_{bs}$ Localized fluorescent spot Chr XV his3:: pYS0046 ($LexA_{bp}$-iRFP720)+ ChrIII URA3:: pYS0074 ($LexA_{bs}$x32) Marker for yeast integration: URA3 (Linearization by BstBI) |

**Table 32:** Strains list-continue

| Strain name | Origin/ Mother strain | Source | Genotype | Description |
|---|---|---|---|---|
| YS0040 | YS0014 | Prof. Shechtman lab | MATα leu2 his3::pKW3034 (HIS3) ade2 trpl ura3 HML::pYS0019 | Fused Fluorescent Repressor LacI-eGFP spread all over the nucleus; Localization of TetR-mCherry in HML region Chr III (15088-15621bp; HML):: pYS0019 (TetO) Chr XV his3Δ1:: pKW3034 (LacI-eGFP & TetR-3*mCherry) Marker for yeast integration: URA3 (Linearization by BmgBI) |
| YS0041 | YS0015 | Prof. Shechtman lab | MATa, ade2-1, can1-100, his3-11::pKW3034 (HIS3), leu2-3, trp1-1, ura3-1 HML::pYS0019 | Fused Fluorescent Repressor LacI-eGFP spread all over the nucleus; Localization of TetR-mCherry in HML region Chr III (15088-15621bp; HML):: pYS0019 (TetO) Chr XV his3Δ1:: pKW3034 (LacI-eGFP & TetR-3*mCherry) Marker for yeast integration: URA3 (Linearization by BmgBI) |
| YS0042 | YS0032 | This research | MATα leu2 his3::pYS0046 ade2 trpl ura3::pYS0047 | $LexA_{bp}$ repressor fused to iRFP720 fluorescent protein& 64 repeats of $LexA_{bs}$ Localized fluorescent spot Chr XV his3:: pYS0046 ($LexA_{bp}$-iRFP720)+ ChrIII URA3:: pYS0047 ($LexA_{bs}$x64) Marker for yeast integration: URA3 (Linearization by BstBI) |
| YS0043 | YA204 | This research | MATα leu2 his3::pYS0075 ade2 trpl ura3 | LacI repressor fused to iRFP720 fluorescent protein Fluorescence all over the nucleus Chr XV his3:: pYS0075 (LacI-iRFP720) Marker for yeast integration: HIS3 (Linearization by NheI-HF) |
| YS0044 | YA204 | This research | MATα leu2 his3::pYS0077 ade2 trpl ura3 | TetR repressor fused to iRFP720 fluorescent protein Fluorescence all over the nucleus Chr XV his3Δ1:: pYS0077 (TetR-iRFP720) Marker for yeast integration: HIS3 (Linearization by NheI-HF) |

**Table 32:** Strains list-continue

| Strain name | Origin/ Mother strain | Source | Genotype | Description |
|---|---|---|---|---|
| YS0045 | YA204 | This research | MATα<br>leu2 his3::pYS0076 ade2 trpl ura3 | λcI repressor fused to iRFP720 fluorescent protein<br>Fluorescence all over the nucleus<br>Chr XV his3Δ1:: pYS0076 (λcI - iRFP720)<br>Marker for yeast integration: HIS3 (Linearization by NheI-HF) |
| YS0046 | YS0045 | This research | MATα<br>leu2 his3::pYS0076 ade2 trpl ura3::pYS0078 | λcI repressor fused to iRFP720 fluorescent protein&λOx64 binding sites<br>Localized fluorescent spot<br>Chr V Ura3-1:: pYS0078 (λOx64)<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| YS0047 | YS0044 | This research | MATα<br>leu2 his3::pYS0077 ade2 trpl ura3::pYS0003 | TetR repressor fused to iRFP720 fluorescent protein&TetOx112 binding sites<br>Localized fluorescent spot<br>Chr V Ura3-1:: pYS0003 (112xTetO)<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| YS0048 | YS0043 | This research | MATα<br>leu2 his3::pYS0075 ade2 trpl ura3::pYS0079 | LacI repressor fused to iRFP720 fluorescent protein&LacOx256 binding sites<br>Localized fluorescent spot<br>Chr V Ura3-1:: pYS0079 (256xLacO)<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| YS0053 | YS0032 | This research | MATα leu2 his3::pYS0046 ade2 trpl ura3::pYS0003 | $LexA_{bp}$ repressor fused to iRFP720 fluorescent protein&112xTetO<br>Chr V Ura3-1:: pYS0003 (112xTetO)<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| YS0054 | YS0032 | This research | MATα leu2 his3::pYS0046 ade2 trpl ura3::pYS0079 | $LexA_{bp}$ repressor fused to iRFP720 fluorescent protein&256xLacO<br>Chr V Ura3-1:: pYS0079 (256xLacO) |

| | | | | |
|---|---|---|---|---|
| | | | | Marker for yeast integration: URA3 (Linearization by BstBI) |

**Table 32:** Strains list-continue

| Strain name | Origin/ Mother strain | Source | Genotype | Description |
|---|---|---|---|---|
| YS0055 | YS0043 | This research | MATα leu2 his3::pYS0075 ade2 trpl ura3::pYS0047 | LacI repressor fused to iRFP720 fluorescent protein&64xLexA binding sites<br>Chr V Ura3-1:: pYS0047 ($64xLexA_{bs}$)<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| YS0056 | YS0044 | This research | MATα leu2 his3::pYS0077 ade2 trpl ura3::pYS0047 | Fused Fluorescent Repressor TetR-iRFP720&$64xLexA_{bs}$ Chr V Ura3-1:: pYS0047 ($64xLexA_{bs}$)<br>Marker for yeast integration: URA3 (Linearization by BstBI) |
| YS0058 | YA204 | This research | MATα leu2 his3::pYS0096 ade2 trpl ura3 | Fused Fluorescent Repressor LexA-iRFP720-NLS-C-Term<br>Marker for yeast integration: HIS3 (Linearization by NheI-HF) |
| YS0059 | YS0040 | This research | MATα leu2 his3::pKW3034 (HIS3) ade2 trpl::pYS0095 ura3 HML::pYS0019 | Fused Fluorescent Repressor LacI-eGFP spread all over the nucleus; Localization of TetR-mCherry in HML region Chr III (15088-15621bp; HML):: pYS0019 (TetO) Chr XV his3Δ1:: pKW3034 (LacI-eGFP & TetR-3*mCherry) Trp1::pYS0095 (LexA-iRFP720-NLS-C-Term)<br>Marker for yeast integration: TRP1 (Linearization by Bsu36I) |
| YS0060 | YS0041 | This research | MATa, ade2-1, can1-100, his3-11::pKW3034 (HIS3), leu2-3, trp1-1::pYS0095, ura3-1 HML::pYS0019 | Fused Fluorescent Repressor LacI-eGFP spread all over the nucleus; Localization of TetR-mCherry in HML region Chr III (15088-15621bp; HML):: pYS0019 (TetO) Chr XV his3Δ1:: pKW3034 (LacI-eGFP & TetR-3*mCherry) Trp1::pYS0095 (LexA-iRFP720-NLS-C-Term)<br>Marker for yeast integration: TRP1 (Linearization by Bsu36I) |

**Table 32:** Strains list-continue

| Strain name | Origin/ Mother strain | Source | Genotype | Description |
|---|---|---|---|---|
| YS0061 | YS0058 | This research | MATα leu2 his3::pYS0096 ade2 trpl ura3::pYS0047 | Fused Fluorescent Repressor Localization of LexA-iRFP720 in URA3 HIS3::LexA-iRFP720-NLS-C-Term (pYS0096) URA3:: LexAx64 binding sites (pYS0047) |
| YS0062 | YS0059 | This research | MATα leu2 his3::pKW3034 (HIS3) ade2 trpl::pYS0095 ura3 HML::pYS0019 HMR::pYS0093 | Fused Fluorescent Repressor LacI-eGFP spread all over the nucleus; Localization of TetR-mCherry in HML region Chr III (15088-15621bp; HML):: pYS0019 (TetO) Chr XV his3Δ1:: pKW3034 (LacI-eGFP & TetR-3*mCherry) Trp1::pYS0095 (LexA-iRFP720-NLS-C-Term), Localization of LexA-iRFP720 in HMR region ChrIII (295192-295337) Marker for yeast integration:ADE2 (Linearization by PspXI) |

# 8 Discussion and summary

It has long been known that the genetic information of all living organisms is encoded in the linear DNA sequence. However, a variety of processes in eukaryote cells require precise chromosome folding which forms a unique topology and plays a crucial role in gene expression and regulation, DNA repair, nuclear transport, and more[64].

Over the years, various technologies have developed to better understand and analyze the spatial organization of chromatin. Chromatin conformation capture (3C) and related techniques (4C, 5C and Hi-C) have revolutionized the analysis of chromosome folding by providing inter-loci spatial proximity maps and revealing important interactions between genomic loci that may be located at a great distance in the linear DNA[5]. While exposing unknown conformational characteristics, these techniques cannot provide dynamic information. Moreover, when comparing DNA FISH with chromosome conformation capture techniques results, discrepancies are sometimes found, probably as a result of the fixation procedure [65].

Tracking chromatin dynamics in 3D in a single live cell provides the most accurate picture of the chromosome conformational changes and requires a specific and non-destructive technique. One technique that fulfills these requirements is the Fluorescent repressor-operator system (FROS) that was first introduced in 1996[11] with the Lac system, followed by the development of other systems based on Tet[22], Lambda[66] and Mal[67].

Here, we developed a new FROS using the bacterial LexA repressor-operator system together with the bright near-infrared fluorescent protein iRFP720 [68], which to our knowledge has never been used before in this approach. This system uses fewer operator repeats compared to other FROS (between 50-65 repeats), thus causes less genome perturbation, with higher localized spot fluorescence intensity and stable signal in *saccharomyces cerevisiae* yeast strains. The plasmid containing the LexA operator array was constructed using a cloning strategy with three different restriction enzymes- PacI, XbaI and AvrII, and by relying on compatible sticky ends generated by XbaI and AvrII, which after ligation generate a restriction site that cannot be recognized by either enzyme. The number of the operator repeats is double with each cloning cycle, while the restriction map remains unchanged. In an attempt to reduce the repetitiveness of the sequence and to prevent the reduction in the number of the operators in bacteria and yeast cells, four different LexA operator libraries and four different oligos containing different combinations of the libraries were designed. Each oligo contained two different restriction sites in the edges that form compatible overhangs to other restriction digestion in another oligo. After ligation, all four oligos were assembled and formed 16 LexA operator repeats. However, the second cycle failed to generate 64 LexA operator repeats.

Since strong fluorescence intensity of a localized spot is highly desirable for high localization precision, this parameter was compared with the other widely used systems- Lac and Tet, by fusing all repressors to iRPF720 and expressing them under the same promoter, and by integrating the

different operator arrays at the same locus (in three different yeast strains) for accurate comparison. Even though LexA system uses fewer operator repeats compared to Tet and Lac, the fluorescence intensity of the localized fluorescent spot was the highest. Furthermore, the fluorescence intensity of the localized LexA-iRFP720 spot was found to be high enough for PSF engineering. However, the fluorescence background (generated by unbound fused repressors) was the highest and hence the signal to background ratio using the LexA systems was the lowest, probably due to more unbound fluorescent repressors compared to Tet and Lac systems. In future work, to reduce the number of unbound repressors, the promoter that controls their expression will be replaced with a weaker promoter.

The specificity of the recognition of each repressor to its operator was tested, by integrating matched and unmatched combinations of a repressor-operator array of LexA, Tet and Lac systems to yeast cells. Only matched combinations (TetR/TetO, LacI/LacO and LexA binding protein/LexA binding sites) formed bright localized spots, while the fluorescence with the unmatched combinations was visualized all over the nucleus, meaning that the recognition is highly specific, and the three systems can be used simultaneously to label three loci using different fluorescent proteins with separate emission spectra.

The new LexA-iRFP720 system was used together with the Tet-mCherry system to label two loci that involve in mating-type switching event (HMRa and HMLα, respectively). Both spots were clearly visualized and spatially separated, however only a small subset out of the entire population was labeled in both loci. In future work, we wish to repeat the integration so that more cells in the population will bear two localized spots and sort the double-labeled cells using FACS sorter. Sorted cells will be propagated and used for further labeling of loci involved in mating type switching by different FROS with different fluorescent proteins. Since the integration of more than three FROS requires the use of a yeast strain bearing at least 7 mutations, the integration of the fourth system will be based on CRISPR/Cas9 instead of chemical transformation.

Finally, we wish to measure the changes in distances between each pair of labeled loci, each with different fluorescent protein, in all three dimensions, to provide quantitative data about the spatial reorganization of the chromatin during a mating type switching event.

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# 10 Appendix

## 10.1 SARS-Cov-2 spike protein expression and purification

One of the main challenges of fighting the SARS-COV2 virus responsible for the ongoing Coronoavirus disease pandemic (COVID19) is the early detection of infection at high throughput, due to the occurrence of asymptomatic infected individuals that spread the disease unknowingly. Besides, early and continuous detection of antibodies is crucial to determine the progression of the disease and future immunity on a population scale. Overcoming the limitations of current genetic or immunoassay tests will affect decision making regarding the worldwide strategy of entire population confinements to prevent the spread of COVID19.

In parallel to the development of a new FROS based on LexA, I took part in a project developing a microfluidics-based approach to detect the presence of single particles, either the virus or antibodies (separately), in the serum of a suspected patient via fluorescence. By combining a commercially available technology for labeling biomolecules in solution, with an advanced microscopy technique developed in our laboratory, 3D multicolor imaging in flow, we plan to detect interactions at very high sensitivity.

For the detection of antibodies, we aim to fluorescently label antibodies in the serum, mix the serum with a fluorescent recombinant spike protein and determine binding by colocalization of the two fluorescent molecules. This approach does not distinguish between antibody isotypes thus will potentially increase sensitivity and provide new serological information.

For this reason, a soluble spike protein (S) and a receptor-binding domain (RBS) fused to His-tag, kindly provided by Krammer lab[69], and Avi-tag at either the C or N-terminal end (that was fused in our lab) with a signal peptide for efficient secretion was expressed and purified from FreeStyle HEK-293F cells or adherent HEK-293 cells.

Adherent HEK-293 cells were transfected as described in section 6.8.4.1 with a plasmid containing a gene that encoding either the soluble spike or the soluble RBD fused to His-tag (Figure 15), after purification with Midiprep plasmid purification kit, while the FreeStyle cells were transfected as described in section 6.8.4.2 with a plasmid containing a gene that encoding the soluble RBD fused to His-tag and Avi-tag at either C or N-terminal (Figure 16). 3 days post transfection, the proteins were purified from the growth medium with a His-tag column as described in section 6.9.1 and the purified protein was run in SDS-PAGE gel (as described in section 6.9.2) to verify that they are in the expected length.

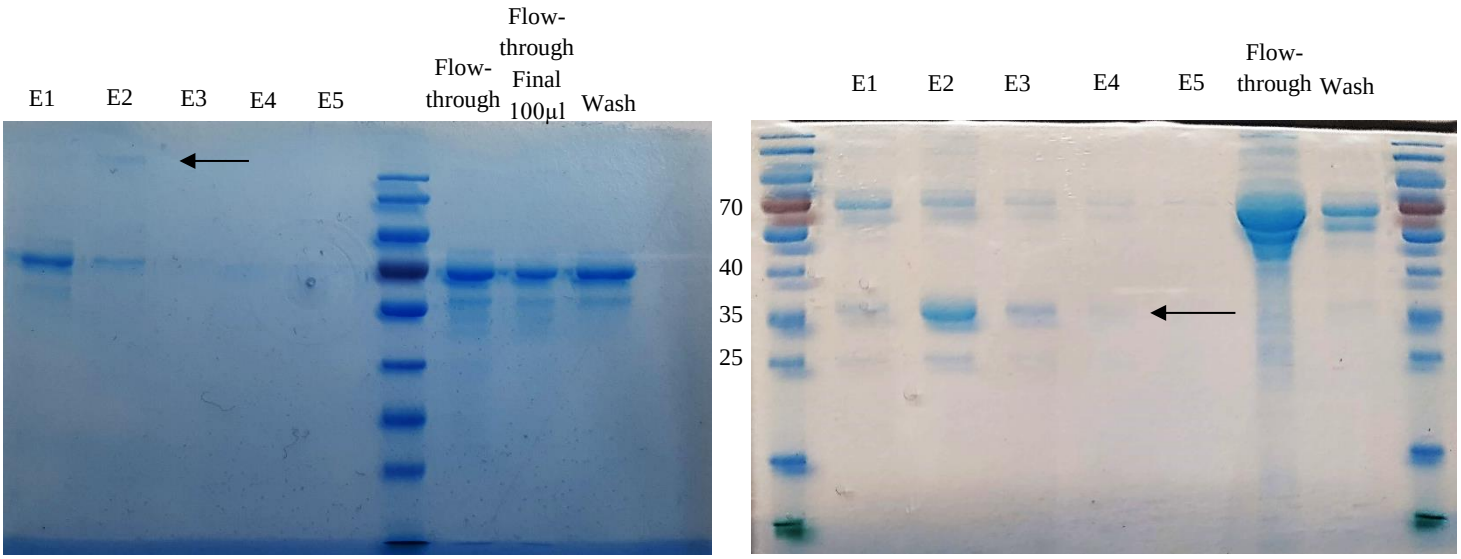


**Figure 15: SDS-PAGE gels (12% acrylamide).** Different fractions (E-eluted protein, Flow-through-unbound proteins, Wash-proteins that were detached from the column after the addition of the wash solution) obtained from protein purification using adherent HEK-293 cells transfected with a plasmid containing a gene that encoding the soluble spike fused to His-tag (left gel) or the soluble RBD fused to His-tag (right gel). The arrows indicate the bands in the expected length (approximately 140kDa for soluble spike and 35kDa for soluble RBD). Protein marker- PageRuler (26616, Thermo Fisher).

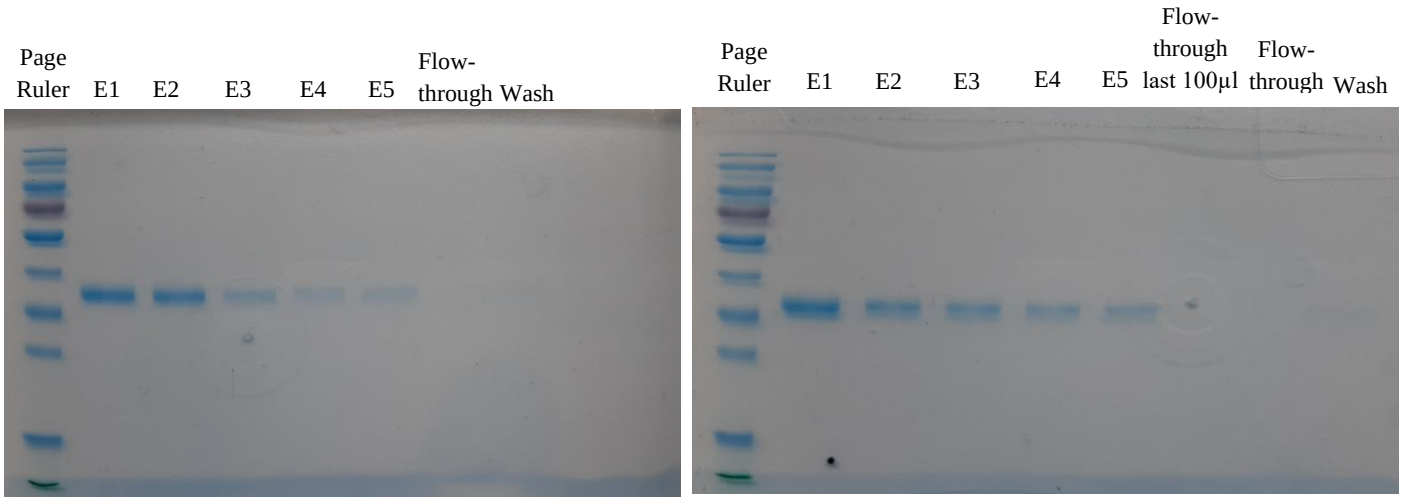


**Figure 16: SDS-PAGE gels (12% acrylamide).** Different fractions (E-eluted protein, Flow-through-unbound proteins, Wash-proteins that were detached from the column after the addition of wash solution) obtained from protein purification using FreeStyle HEK-293F cells transfected with a plasmid containing a gene that encoding the soluble RBD fused to His-tag and Avi-tag at the C-terminal (left gel) or at the N-terminal (right gel). The bands are in the expected length (approximately 35kDa). Protein marker- PageRuler.

As can be seen in Figure 15, the expected lengths of both the soluble spike and soluble RBD fused to His-tag were obtained, but in low concentration and together with other undesired proteins that are probably originated from the serum that was added to the growth medium. However, purification of proteins expressed by FreeStyle cells produced only single desired band in a high concentration (Figure 16), since FreeStyle cells are optimized for protein expression and their growth medium do not contain serum.

## 10.2 Primers / Oligos list

| # | Primer/oligo name | Primer sequence (5'-3') |
|---|---|---|
| 1 | F_iRFP720_Insert_Gibson | ccaagcccgctcatgatcatggcggaaggatccgtc |
| 2 | R_iRFP720_Insert_Gibson | gtcgacggtatcgataagcttttactcttccatcacgccg |
| 3 | F_iRFP720_BB_Gibson | cggcgtgatggaagagtaaaagcttatcgataccgtcg |
| 4 | R_iRFP720_BB_Gibson | gacggatccttccgccatgatcatgagcgggcttgg |
| 5 | R_Characterization_BB | ccgggggatccactagttgttaattcag |
| 6 | F_Characterization_BB | ggttctaaggatatctctgctggag |
| 7 | F_TetR_iRFP720_Insert | ctgaattaacaactagtggatcccccggatgccaaagaagaagcgtaaggtctc tag |
| 8 | R_TetR_iRFP720_Insert | ctccagcagagatatccttagaacctccacttggagcggatcccc |
| 9 | F_Lambda_iRFP720_Insert | ctgaattaacaactagtggatcccccggATGAGCACAAAAAAG AAACCATTAACACAAGAGCAGC |
| 10 | R_Lambda_iRFP720_Insert | ctccagcagagatatccttagaaccGCCAAACGTCTCTTCAGG CCACTGAC |
| 11 | F_LacI_iRFP720_Insert | ctgaattaacaactagtggatcccccggatggtgaaatatgtaacgttatacgatg tcgcagag |
| 12 | R_LacI_iRFP720_Insert | ctccagcagagatatccttagaacccagctgcattaatgaatcggccaacg |
| 13 | R_LacO_BB_SphI | gatattgcatgcgctgtttctccaattcgc |
| 14 | F_LacO_BB_SpeI | ctatacttctatactagtggatcccccg |
| 15 | F_Protein_iRFP720_Seq | ggatcttctactacatcagctt |
| 16 | F_Ura3_Verification | GGTGAAGGATAAGTTTTGACCATCAAAGAAGG |
| 17 | F_URA3_LacO_PfoI | gtacctaTCCGGGAttagttttgctggccgcatcttctcaaatatg |
| 18 | R_URA3_LacO_AatII | gatcggaGACGTCctgtggtttcagggtccataaagcttttcaattc |
| 19 | F_gRNA_Chr.XV_iRFP720 | GCTTTAACCGATCTATTCATGTTTT |
| 20 | R_gRNA_Chr.XV_iRFP720 | ATGAATAGATCGGTTAAAGCGATCA |
| 21 | F_gRNA_HMR_LexAx64 | GGAAAAAACGCTAAAGTGTGGTTTT |
| 22 | R_gRNA_HMR_LexAx64 | CACACTTTAGCGTTTTTTCCGATCA |
| 23 | Oligo R | GCAGCGTTCAAACCGCTAACGATCA |
| 24 | T7 Primer | TAATACGACTCACTATAGGG |
| 25 | F_LexA_iRFP720_Cas9_New | CTATATTTTCCACTATTTTTGCTTAATTGCTTTAA CCGATCTATTgatgtgctgcaaggcgattaagttgggtaacgcc |
| 26 | R_LexA_iRFP720_Cas9_New | GAAGTGGAAATAAAAGATAATGTAAATAATCAG TCCAATGcttttgctggccttttgctcacatgttctttcc |
| 27 | F_pSR6_URA3_colonyPCR | ggttaatgtggctgtggtttcagggtccataaagc |
| 28 | R_pSR6_URA3_colonyPCR | cagtttttttagttttgctggccgcatcttctc |
| 29 | F_Seq_gRNA_bRA66 | gtaatacgactcactatagggcgaattggagctc |
| 30 | R_Trp1_BB_LexA_iRFP720 | GGA AAG GAC TGT GTT ATG ACT TCC CTG AC |
| | | |

| **Primers / Oligos list- continue** | | |
|---|---|---|
| **#** | **Primer/oligo name** | **Primer sequence (5'-3')** |
| 31 | F_Trp1_Insert_LexA_iRFP720 | GTC AGG GAA GTC ATA ACA CAG TCC TTT CCC GTG ATT AAG CAC ACA AAG GCA GCT TGG |
| 32 | F_Trp1_BB_LexA_iRFP720 | GAC AAG GTA ATG CAT CAT TCT ATA CGT GTC |
| 33 | R_Trp1_Insert_LexA_iRFP720 | GAC ACG TAT AGA ATG ATG CAT TAC CTT GTC CTT GCT TTT CAA AAG GCT TGC AGG CAA G |
| 34 | F_LexA_Trp1_open | GAACTCGATTTCTGACTGGGTTGGAAGGCAAG |
| 35 | R_LexA_Trp1_open | CAATCCAAAAGTTCACCTGTCCCACCTGC |
| 36 | F_HR_HMR_LexAx64_XmaI | ctaagtcacccgggcagtatatagaaatatagattccctttgaggattcctatatcc |
| 37 | R_HR_HMR_LexAx64_PstI | ctgacttagctgcagtgcagatgcggaattggtgaatttttatattattgtgggg |
| 38 | F_ADE2_LexAx64_PfoI | gctaatcTCCCGGAGACGGTCACAGCTTGTCTGTAAG |
| 39 | R_ADE2_LexAx64_PacI | catcccttaattaaCAATTTCCTGATGCGGTATTTTCTCCTTACGC |
| 40 | F_Insert_iRFP720_NLS_C | ggccaagcccgctcatgatcatggcggaaggatccgtc |
| 41 | R_Insert_iRFP720_NLS_C | ttaggcaacctttctcttcttctttggtggagtacaggatcccagctcttccatcacgccgatctg |
| 42 | R_BB_iRFP720_NLS_C | gatcatgagcgggcttggcc |
| 43 | F_BB_iRFP720_NLS_C | ctgggatcctgtactccaccaaagaagaagagaaaggttgcctaaaagcttatcgataccgtcgacctcgag |
| 44 | F_ColonyPCR_ADE2_LexAx32 | ATGGATTCTAGAACAGTTGGTATATTAGGAGGGG |
| 45 | R_ColonyPCR_ADE2_LexAx32 | GACACCTGTAAGCGTTGATTTCTATGTATG |
| 46 | F_ColonyPCR_NLS_C_Term | ctgcgacgatgagccgatccatatc |
| 47 | R_ColonyPCR_NLS_C_Term | caacctttctcttcttctttggtggagtacagg |
| 48 | F_ColonyPCR_NLS_N_Term | atgccaaagaagaagcgtaaggtc |
| 49 | R_ColonyPCR_NLS_N_Term | ctccagcagagatatccttagaacc |
| 50 | F_ColonyPCR_HR_HMR | gattccctttgaggattcctatatcctcg |
| 51 | R_ColonyPCR_HR_HMR | ccataacctaaagtagtgactaaggttggcc |
| 52 | F_Seq_NLS_N_Term | ctgaattaacaactagtggatcccc |
| 53 | R_Seq_NLS_C_Term | cctcactaaagggaacaaaagctgggtac |

| **Primers / Oligos list- continue** | | |
|---|---|---|
| **#** | **Primer/oligo name** | **Primer sequence (5'-3')** |
| 54 | F_PCR_HR_HMR_LexAx64_Start | gacataaggtatgaaaatttgtcaacgaagttagagaaagctggatgcaagg |
| 55 | R_PCR_HR_HMR_LexAx64_Start | ctgggtaccaagcagacgctactaaggaaaacaacaacggaagcgatgatg |
| 56 | F_Seq_HR_HMR_LexAx64_Start | ggaaaatgaaacatataacggaatgagg |
| 57 | F_PCR_HR_HMR_LexAx64_End | cattaggacctttgcagcataaattactatacttctatactagtggatcccccg |
| 58 | R_PCR_HR_HMR_LexAx64_End | cggttgaataaacctggtctcaaataaaattggtagaatgacctagaatgaccca tccg |
| 59 | F_Seq_HR_HMR_LexAx64_End | gtatatagaaatatagattccctttgaggattcc |

# פיתוח מערכת מעכב-אופרטור פלואורסנטית המבוססת על LexA לצורך סימון דנ"א בתאים חיים

חיבור על מחקר

לשם מילוי חלקי של הדרישות לקבלת התואר מגיסטר למדעים בהנדסת ביוטכנולוגיה ומזון

נועה נבון

הוגש לסנט הטכניון-מכון טכנולוגיה לישראל

טבת תשפ"א, חיפה, דצמבר 2020

המחקר נעשה בהנחיית פרופ׳ רועי עמית בפקולטה להנדסת ביוטכנולוגיה ומזון ופרופ׳ יואב שכטמן בפקולטה להנדסה ביו-רפואית.
אני מודה לטכניון - מכון טכנולוגי לישראל, ולמועצה האירופית למחקר (ERC) על התמיכה הכספית הנדיבה בהשתלמותי.

# 1 תקציר


מעבר למידע הגנטי המקודד בדנ"א הליניארי, המבנה המרחבי של הכרומטין- כלומר הגנום והביו-מולקולות הקשורות אליו, ממלא תפקיד חשוב בבקרה של מגוון רחב של תהליכים בתאים אאוקריוטים, ביניהם בקרה על ביטוי גנים, תיקון שברים בדנ"א, בקרה על חלוקה תקינה של התא ועוד.

הארגון המרחבי של הדנ"א בתאים מקובעים נחקר באמצעות מגוון שיטות ביוכימיות, ביניהן שיטת Hi-C, אשר מאפשרת לגלות אינטראקציות בין אזורים שונים בגנום שלעיתים מרוחקים מאוד בדנ"א הליניארי. אמנם שיטה זו והשיטות הדומות לה (3C, 4C, 5C) חשפו אינטראקציות שלא היו ידועות בעבר, אך לא ניתן ללמוד באמצעותן על הדינמיקה של הדנ"א בתאים חיים במהלך תהליכים שונים.

במהלך השנים פותחו מספר שיטות סימון פלואורסנטי למיקרוסקופית אור על מנת לחשוף תהליכים דינמיים הקשורים בדנ"א שהיו חבויים בעבר ומתאימים לשימוש בתאים חיים, ביניהן מערכות המבוססות על המערכת החיידקית CRISPR-Cas9, בהן משתמשים בחלבון Cas9 מוטנטי (dCas9) כך שהיכולת שלו לקשור רצפי דנ"א ספציפיים נותרה, אך ללא היכולת לחתוך את הדנ"א. על ידי חיבורו לחלבון פלואורסנטי או הוספה של אתרי קישור למולקולת הgRNA המזוהים על ידי חלבונים פלואורסנטיים, ניתן לסמן אזורים ספציפיים בגנום. במערכות המבוססות על CRISPR-dCas9 שימוש נרחב, אך הן דורשות תכנון מורכב ולעיתים קרובות גורמות לסימון לא ספציפי. שיטה נוספת המאפשרת סימון ספציפי בתאים חיים מבוססת על מערכת מעכב-אופרטור פלואורסנטית (FROS) שבה חלבונים מעכבים המאוחים לחלבונים פלואורסנטיים קושרים באופן ספציפי מערך של רצפי דנ"א, הנקראים אופרטורים, אשר עברו אינטגרציה לגנום באזור הרצוי. לאחר הקישור, מתקבלת נקודה פלואורסנטית באזור ספציפי בגנום שניתן לעקוב אחר הדינמיקה שלה תחת מיקרוסקופ פלואורסנטי. בשיטה זו נעשה שימוש נרחב לצורך סימון אזורים בגנום בתאי שמר, בין היתר לצורך סימון אזורים המעורבים בתהליך החלפת מין השמר. עד כה היו קיימות שלוש מערכות עיקריות בהן נעשה שימוש בשמרים, הנקראות Tet, Lac ו-Lambda. פיתוח של מערכת סימון נוספת יאפשר בין היתר סימון של ארבעת האזורים העיקריים המעורבים בתהליך ובכך יאפשר למידה נרחבת יותר על הדינמיקה של הדנ"א במהלך ההחלפה, וכן סימון אזורים נוספים המעורבים בתהליכים שונים בתא.

מחקר זה התמקד בפיתוח מערכת סימון חדשה (FROS) שתאפשר לצפות בדינמיקה של לפחות ארבעה לוקוסים (אזורים) שונים בגנום בתלת מימד בתא חי בודד, תוך שימוש בשיטת מיקרוסקופיה מתקדמת המכונה עיצוב חזית גל (Point Spread Function Engineering). בשיטה זו התמונה המתקבלת מצילום מקור האור במיקרוסקופ אופטי משתנה, כך שהיא מקודדת לעומק או לצבע של המקור, וזאת על ידי הרחבה של המסלול האופטי של המיקרוסקופ והוספה של אלמנט אופטי המכונה מסכת פאזה (Phase mask). השיטה מאפשרת מעקב אחר אזורים שונים, המסומנים פלואורסנטית, במקביל בתלת מימד על ידי צילום תמונות בדו מימד ללא צורך בסריקה תלת מימדית הדורשת זמן רב, ובכך משמשת כלי אידיאלי למעקב אחר דינמיקה מהירה בתא חי בודד בתלת מימד.

המערכת החדשה מבוססת על הזיהוי בין המעכב החיידקי LexA, שאוחה לחלבון הפלואורסנטי iRFP720, לאופרטור LexA. המערכת מאפשרת סימון של הדנ"א עם יחס סיגנל לרקע גבוה, מה שמאפשר את השימוש שלה בשיטות הדמיה בסופר-רזולוציה, כגון PSF engineering. המערכת מכילה מערך קטן יותר של רצפי דנ"א בהשוואה לשתי המערכות הנפוצות- Tet ו-Lac, ולכן גורמת פחות הפרעה לגנום השמר, וכן סיגנל פלואורסנטי גבוה יותר באזור הסימון. נעשה שימוש במערכת החדשה בשילוב עם המערכת המבוססת על הזיהוי בין המעכב TetR לאופרטור TetO על מנת לסמן בו זמנית שני אזורים שונים בדנ"א המעורבים בתהליך של החלפת מין תא השמר בשמרי *saccharomyces cerevisiae*.