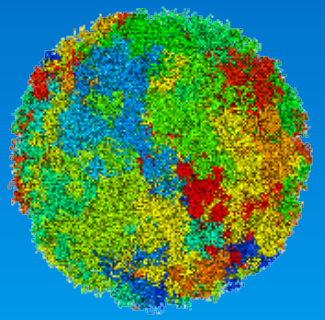

“GFP Walking”

-

Artificial Construct Conversions

Caused by Simultaneous Co-Transfection

Analysed

by

Spatially Resolved Planeometric Microscop

(SIRPM)

by

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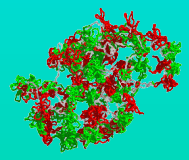
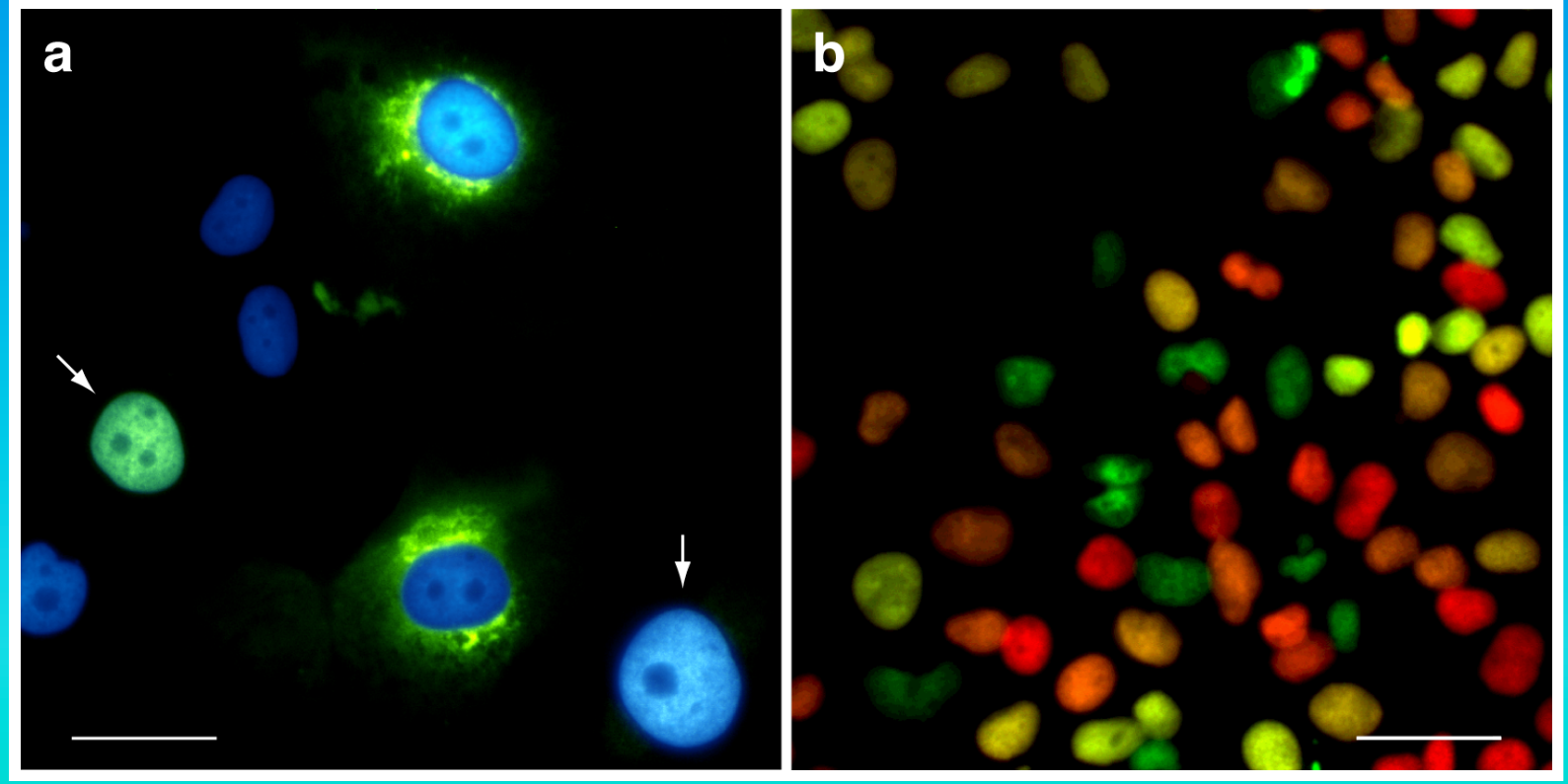


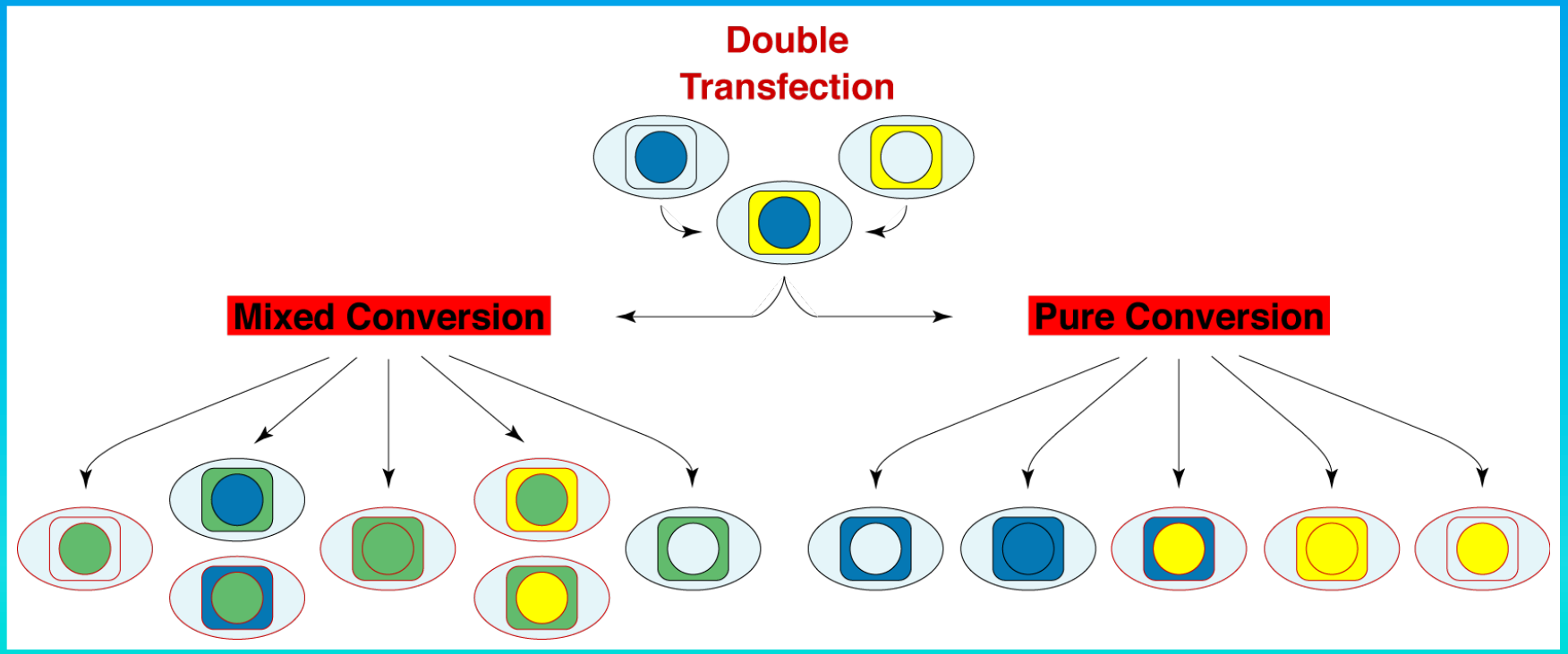
Appearance of Construct Conversion

Simultaneous double transfection of H2A-eCFP and CAT-eYFP into LCLC103H cells leads to stable expression of constructs with altered spectral properties, e. g. H2A-eYFP, with corresponding localization in a considerable number of transfected cells.



Complexity of Possible False-Positive Phenotypes

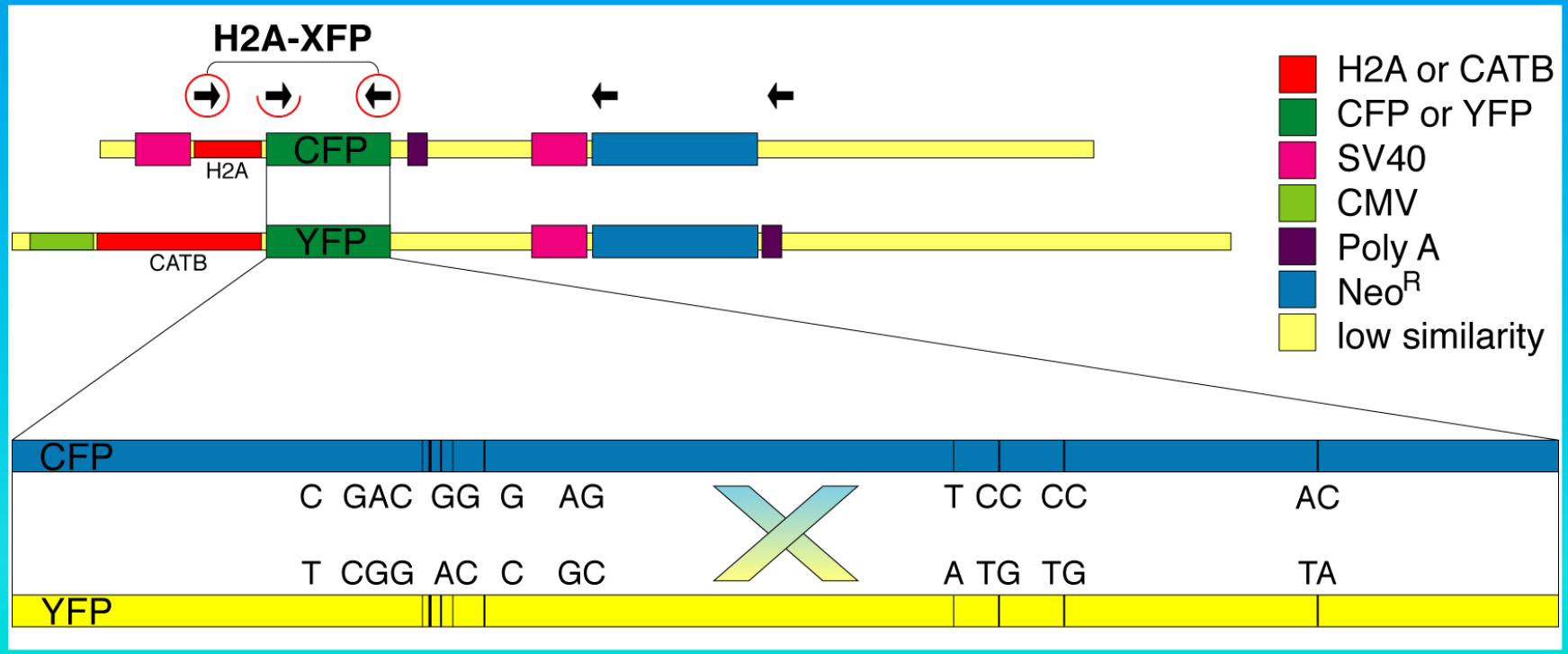
All possible phenotypes caused by simultaneous co-transfection were found in the cell population including mixed conversion, i. e. expression of the correct and altered fusion protein (H2A-eCFP and H2A-eFP).



Sequence Comparison of Vectors

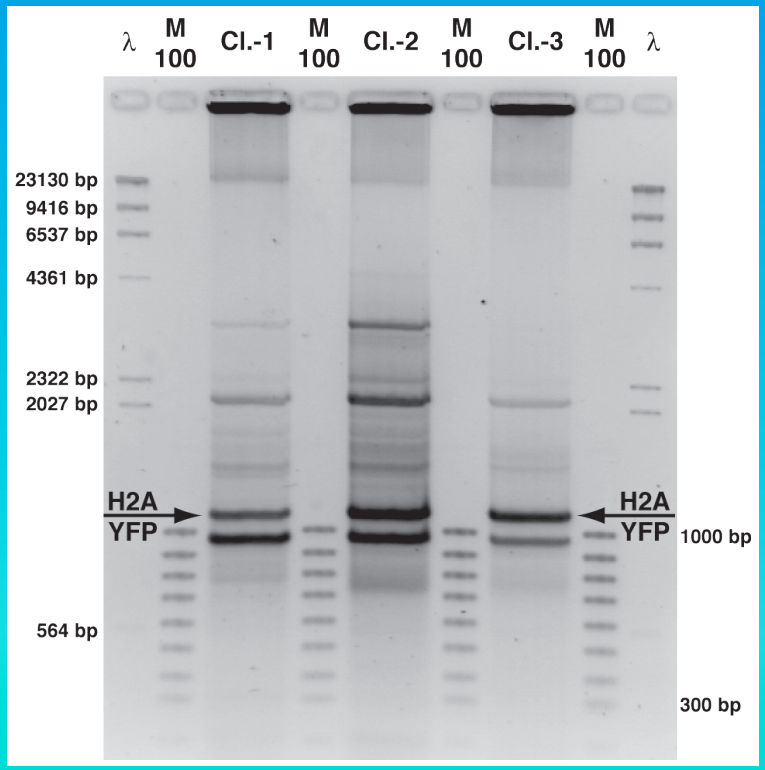
Comparison of the vectors pSV-H2A-eCFP and pcDNA3-CATB-eYFP reveals not only small nucleotidic difference of 16 bp among eCFP and eYFP but also the homologies of the Neo resistance and the SV40 promoters.

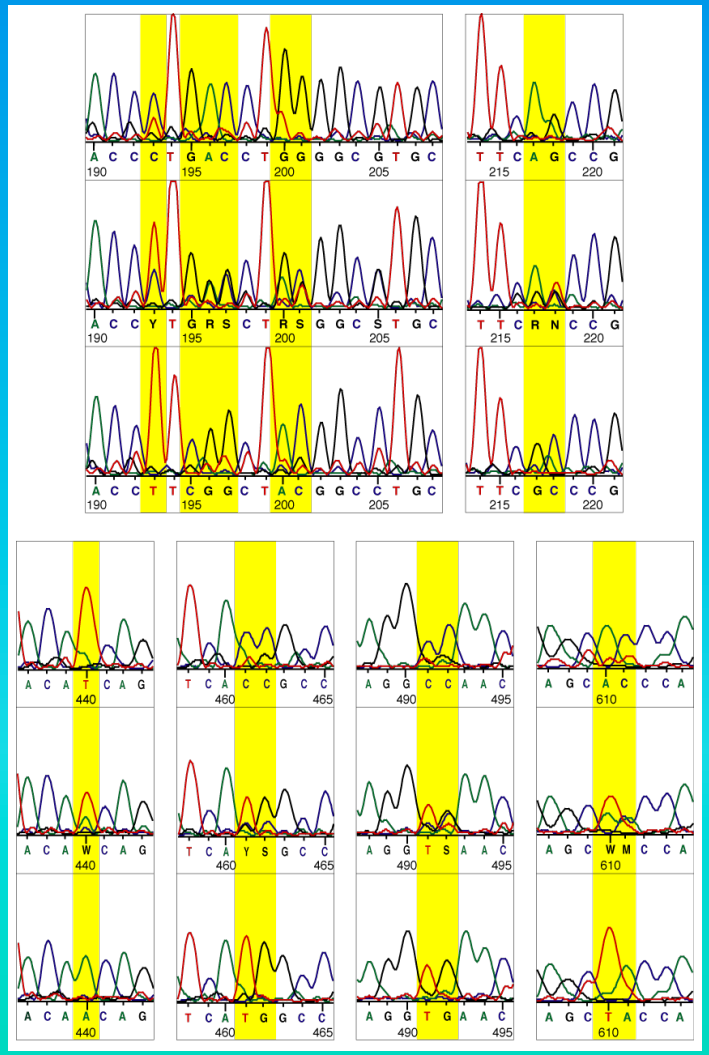
Arrows indicate the position of some of the primers used for genomic PCR to proof conversion.



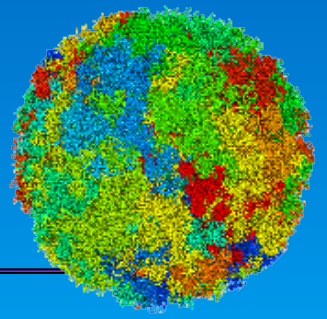
Proof of Conversion on the DNA Sequence Level

Amplification of H2A-eYFP by genomic PCR, gel separation of the product and final sequencing proofs that conversion took place in converted clones.



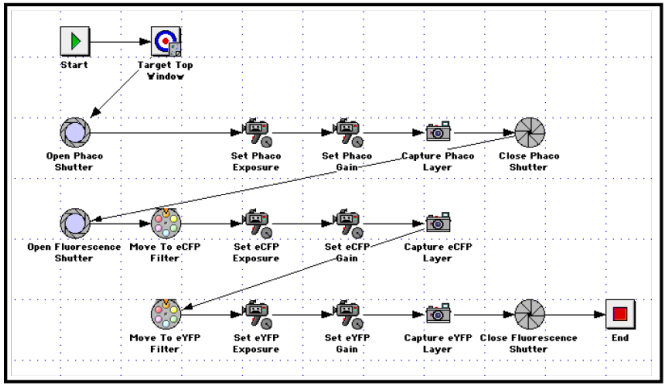


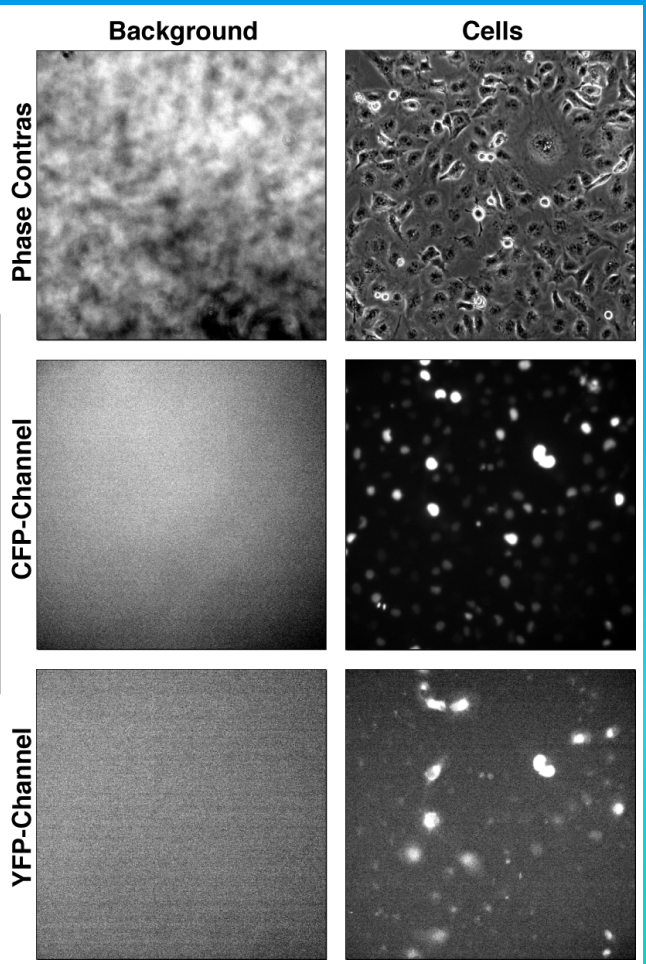
SIRPM - Automated Image Acquisition

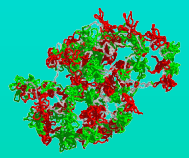


To analyze quantitatively the conversion rates an automated Spatial and Intensity Resolved Planeometric Microscopy (SIRPM) approach was developed.

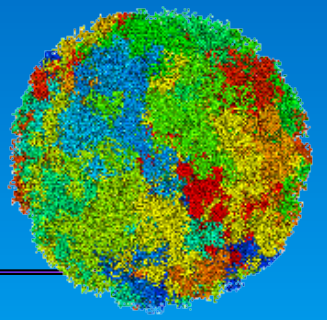
In the first stage images of the cells were acquired automatically in the phase contrast, CFP and YFP channels regarding using an inverse Zeiss microscope controlled by OpenLab (Improvision) software..





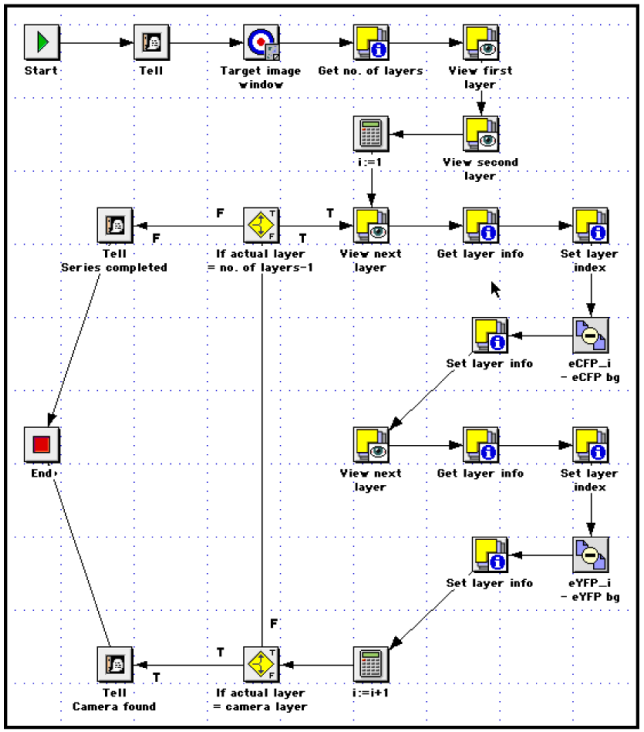


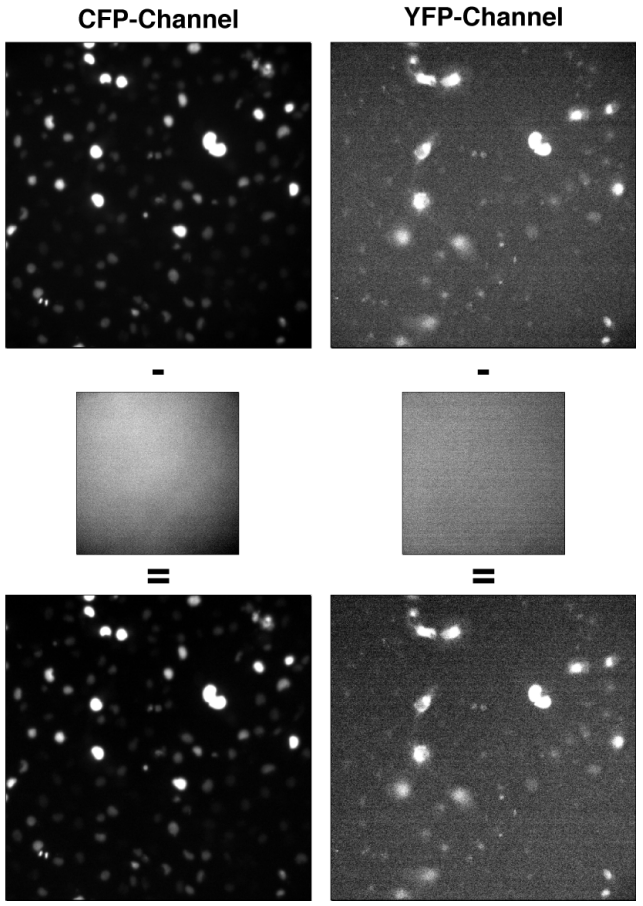
SIRPM - Image Analysis: Background Subtraction

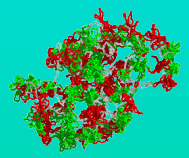


To correct for inhomogeneous illumination and wave front distortions the background has to be subtracted to assure proper results of SIRPM for S+I segmentation.

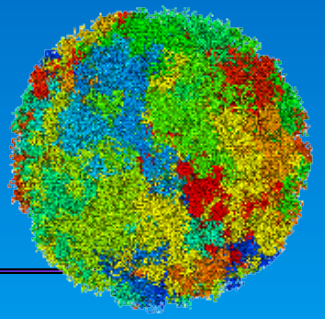
This was done automatically by set-up of a macro using the OpenLab (Improvision) software.





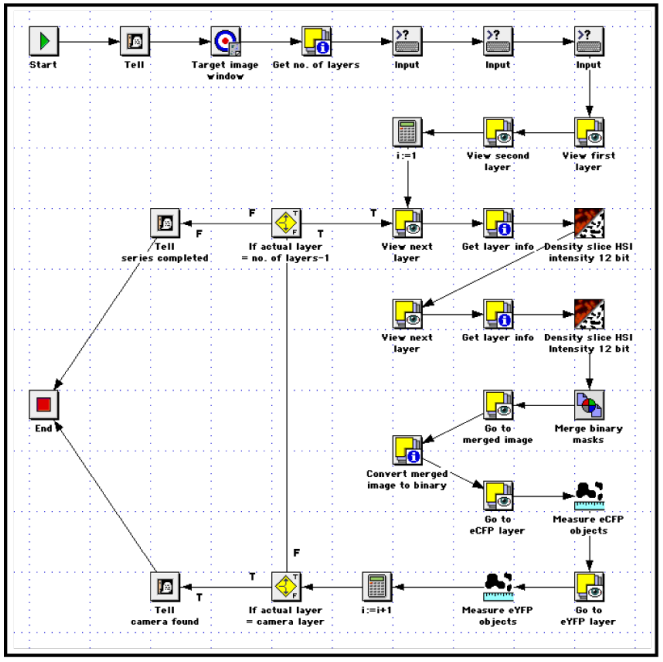


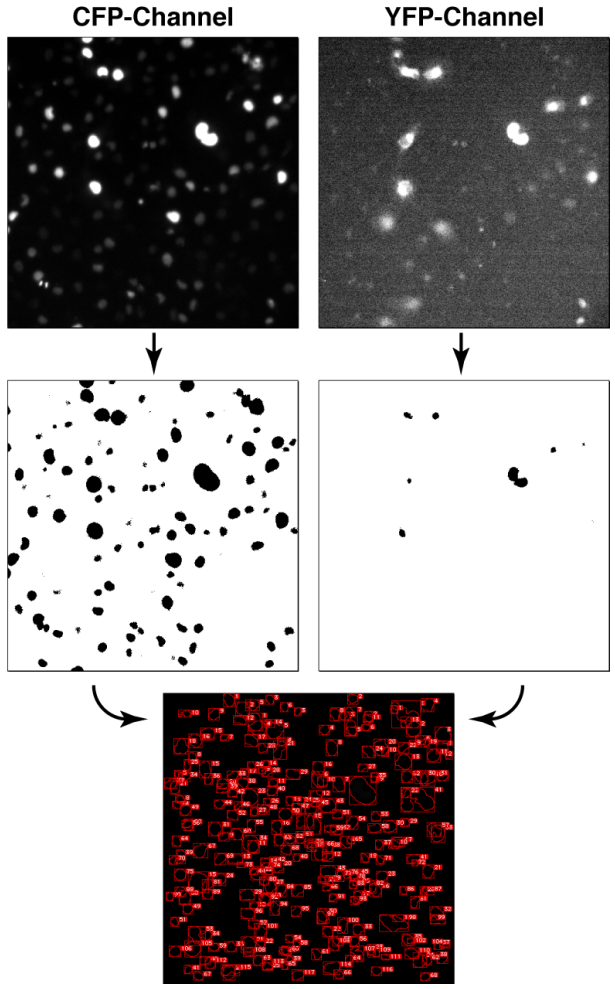
SIRPM - Image Analysis: I+S Segmentation

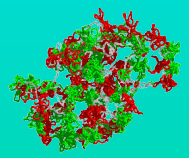


To quantify the number of nuclei in the eCFP and the eYFP channel, the nuclei were segmented first by an intensity threshold applied to the channels. From this a binary mask was created from which the nuclei were further segmented by an area threshold from the CATB signals and background noise.

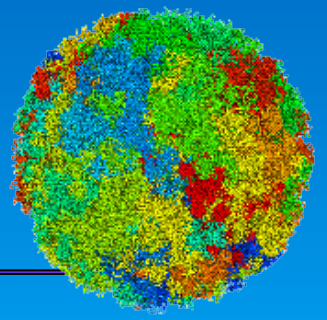
This was done automatically by set-up of a macro using the OpenLab (Improvision) software.





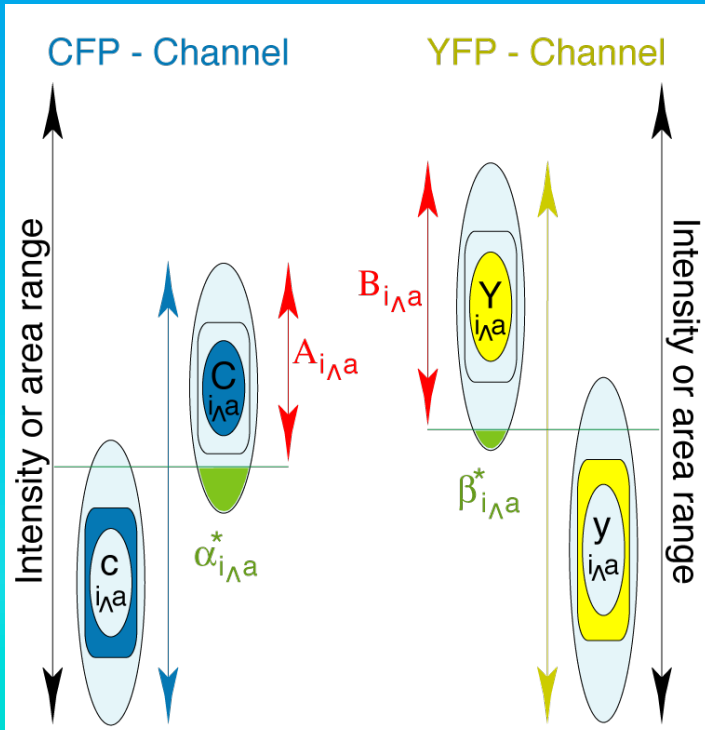


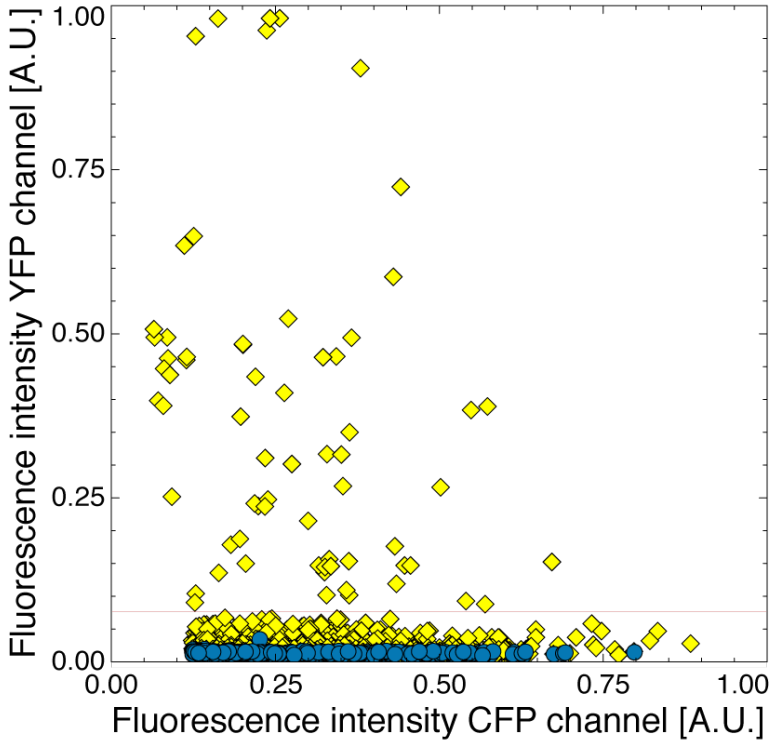
SIRPM - Quantification of the Conversion Rate

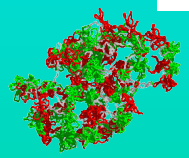


To quantify the total conversion rate correctly, the H2A signals, i. e. the nuclei need not only be imaged correctly regarding the different acquisition of the channels but also need to be segmented by their intensity and area adequately from the CATB and background signals.

$$R_{H2A} = \frac{B_{i \wedge a}}{A_{i \wedge a} + B_{i \wedge a} - (A_{i \wedge a} \wedge B_{i \wedge a})}$$

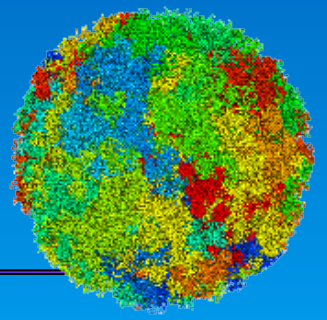






$$R_{total} = 2R_{H2A} - R^2_{H2A}$$

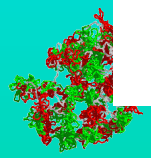
Conversion Rates under Different Conditions



Conversion appears regardless of the cell type and transfection method reaching a total conversion rate of ~8% in our standard approach but could also reach the theoretic limit of 25%!.

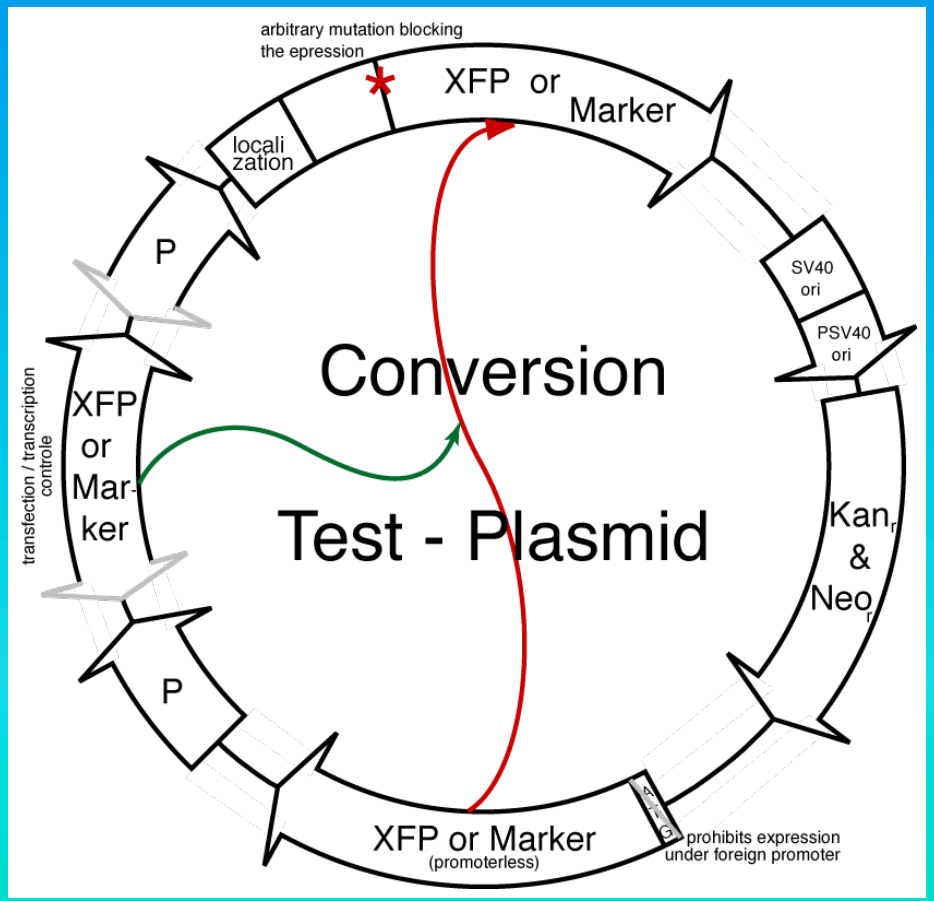
Consequently, "GFP-Walking" not only is a non-neglectable effect but also opens the possibility to investigate replication/repair/recombination (RRR) processes.

Construct	Cells	Method		Conversion [+/-] and [%]	
H2A-CFP	LCLC103H	FuGene6		-	0.0
CB-YFP	LCLC103H	FuGene6		-	0.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	simultanious	++	8.2
H2A-CFP + DsRed	LCLC103H	FuGene6	simultanious	-	0.0
H2A-CFP + pure GFP	LCLC103H	FuGene6	simultanious	+	>2.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	sep. Mix + simultaneous	+	>2.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	sep. Mix + 4 h delay	- (+)	2.0
H2A-CFP* + CB-YFP	LCLC103H	FuGene6	overtransfec. *stable line	-	0.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	5x DNA conc	++	n.q.
H2A-CFP + CB-YFP	LCLC103H	FuGene6	10x DNA conc	++	n.q.
H2A-CFP + CB-YFP	LCLC103H	FuGene6	linearized	+	6.7
H2A-CFP + CB-YFP	LCLC103H	FuGene6	linearized + 96°C	+++	26
H2A-CFP + CB-YFP	LCLC103H	Dmrie-C	simultanious	+	7.5
H2A-CFP + CB-YFP	LCLC103H	Cellfectin	simultanious	+	>2.0
H2A-CFP + CB-YFP	LCLC103H	Lipofectin	simultanious	+	>4.7
H2A-CFP + CB-YFP	LCLC103H	GibcoPlus	simultanious	+	>2.0
H2A-CFP + CB-YFP	LCLC103H	Electroporation	simultanious	+	~2.0
H2A-CFP + CB-YFP	LCLC103H	Ca-Phosphat	simultanious	+	~2.0
H2A-CFP	HeLa	FuGene6		-	0.0
H2A-CFP + CB-YFP	HeLa	FuGene6	simultanious	+	n.q.
H2A-CFP	Cos-7	FuGene6		-	0.0
H2A-CFP + CB-YFP	Cos-7	FuGene6	simultanious	+	n.q

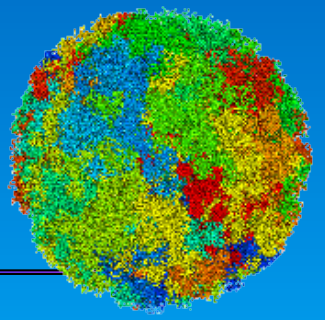


High-Throughput Conversion Analysis

To allow the integral *in vitro* and *in vivo* investigation of replication/repair/recombination (RRR) processes a vector containing functional and non-functional (fluorescent) markers including an internal standard can be constructed for high-throughput screening especially for use in FACS analysis.



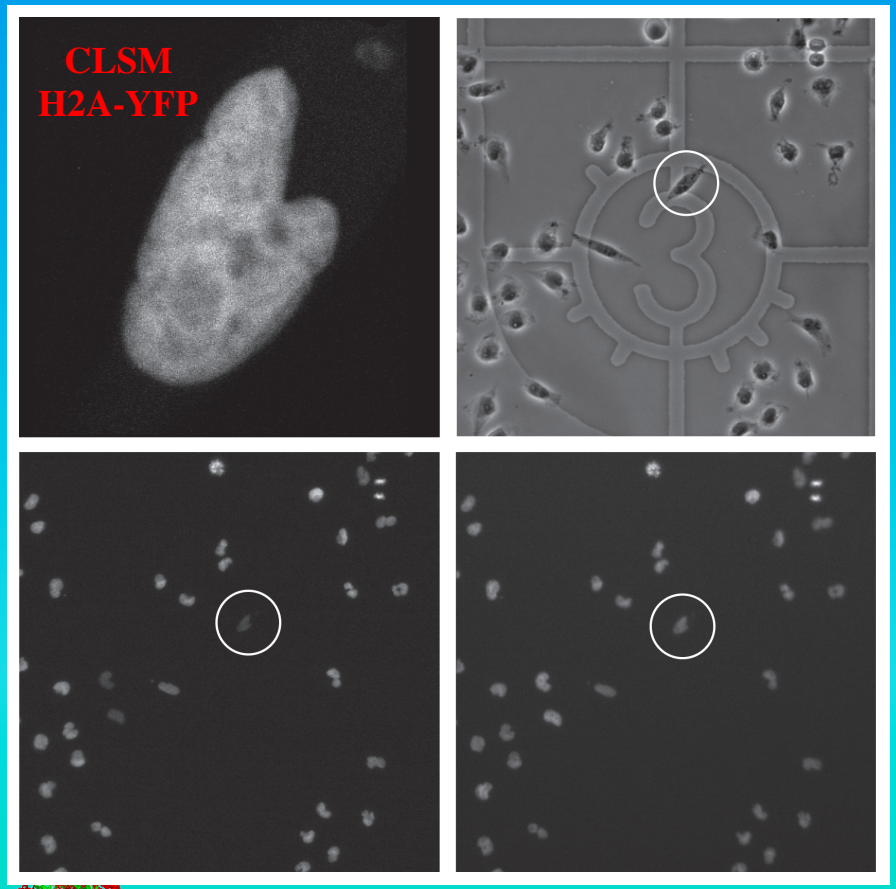
SIRPM for Cell Cycle Analysis on Slide (CCAS)

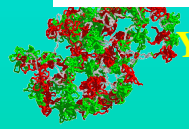


A major problem during experiments as e. g. the analysis of the chromatin distribution *in vivo* by involving individual cells is their unknown cell cycle status.

After DAPI staining and SIRPM the DAPI intensity distribution reveals the classic FACS cell cycle distribution. Thus, the DNA content and thus cell cycle status of an individual cell can be retrieved.

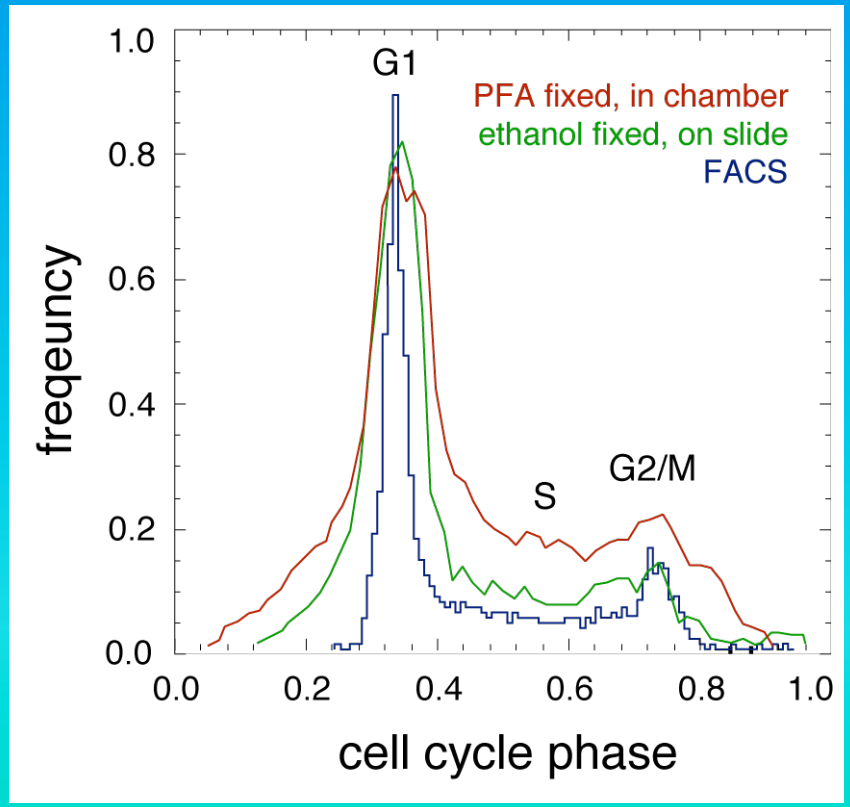
phase contrast





YFP channel

DAPI channel

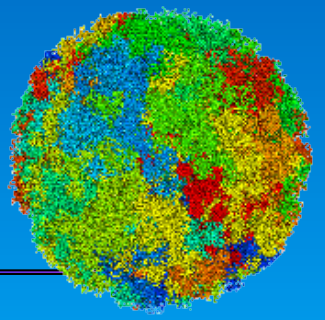


Conclusion

"GFP-Walking" is an important as well as useful effect.

SIRPM is an useful approach.

Acknowledgements



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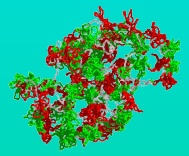

„GFP-Walking“: Artifical Construct Conversions

caused by

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Spatially and Intensity Resolved Planeometric Microscopy (SIRPM)

Knoch, T. A., Bestvater, F. & Spiess, E.

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Abstract

Several GFP variants have been developed for multicolor labeling *in vivo*. Here we report that simultaneous co-transfection of fluorescent protein chimeras can give falsepositive results caused by the conversion of spectral properties. The cDNA of the cysteine protease cathepsin B (CB) tagged with the enhanced yellow fluorescent protein (eYFP) and the cDNA of the histone H2A was tagged with the enhanced cyan fluorescent protein (eCFP) and were cotransfected into lung carcinoma cells. Stable clones with converted fluorescence properties were established by G418 selection and prooven on the DNA sequence level by genomic PCR. Thus, conversion is based on homologous recombination/repair/replication processes that occur between the nucleotide sequences of the fluorescent proteins. To quantify the abundance of conversion high-throughput spatially and intensity resolved planeometric microscopy (SIRPM) was applied: The fluorescent nuclei imaged with an epifluorescence microscope were segmented according to their spatial and intensity properties in both the eCFP and eYFP channels and the conversion rate was calculated from the respective number of fluorescent nuclei. Under standard transfection conditions, approximately 8% of cells produce false-positive results, but, depending on the conditions, up to 26% of the cells permanently express altered fusion proteins. The conversion is independent of transfection methods or cell types. Generally, this compromises the interpretation of results obtained by dual-colour cotransfection using auto-fluorescent proteins. Consecutive transfection or low sequence similarities, however, avoided recombination. The appearance of conversion facilitates exchanges of spectral properties in fusion proteins, the creation of libraries, or the assembly of DNA fusion constructs *in vivo*. The detailed quantification of the conversion rate allows the investigation of recombination/repair/ replication processes in general.

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Keywords:

Genome, genomics, genome organization, genome architecture, structural sequencing, architectural sequencing, systems genomics, coevolution, holistic genetics, genome mechanics, genome function, genetics, gene regulation, replication, transcription, repair, homologous recombination, simultaneous co-transfection, cell division, mitosis, metaphase, interphase, cell nucleus, nuclear structure, nuclear organization, chromatin density distribution, nuclear morphology, chromosome territories, subchromosomal domains, chromatin loop aggregates, chromatin rosettes, chromatin loops, chromatin fibre, chromatin density, persistence length, spatial distance measurement, histones, H1.0, H2A, H2B, H3, H4, mH2A1.2, DNA sequence, complete sequenced genomes, molecular transport, obstructed diffusion, anomalous diffusion, percolation, long-range correlations, fractal analysis, scaling analysis, exact yard-stick dimension, box-counting dimension, lacunarity dimension, local nuclear dimension, nuclear diffuseness, parallel super computing, grid computing, volunteer computing, Brownian Dynamics, Monte Carlo, fluorescence in situ hybridization, confocal laser scanning microscopy, fluorescence correlation spectroscopy, super resolution microscopy, spatial precision distance microscopy, autofluorescent proteins, CFP, GFP, YFP, DsRed, fusionprotein, in vivo labelling.

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