GFP-'Walking': Artificial Construct Aberrations

Caused by Cotransfectional Homologous Recombination

Felix Bestvater

Eberhard Spiess

Biomedical Structure Analysis

Tobias A. Knoch

**Jörg Langowski**

Biophysics of Macromolecules

**German Cancer Research Center (DKFZ)**

Heidelberg - Germany

Simultanious cotransfection of GFP-chimeras lead to a GFP conversion:
All conversion possibilities were observed by cotransfecting
H2A-CFP (SV40, strong expression, localized in the nucleus) and
CB-YFP (CMV, weak expression, localized in the ER/Golgi).
The convertants can be enriched and stable cell lines can be created.

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Double Transfection

Pure Conversion

Mixed Conversion

20 m

50 µm
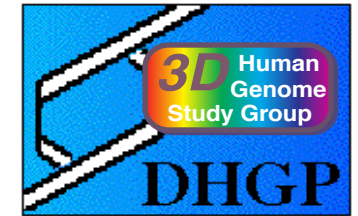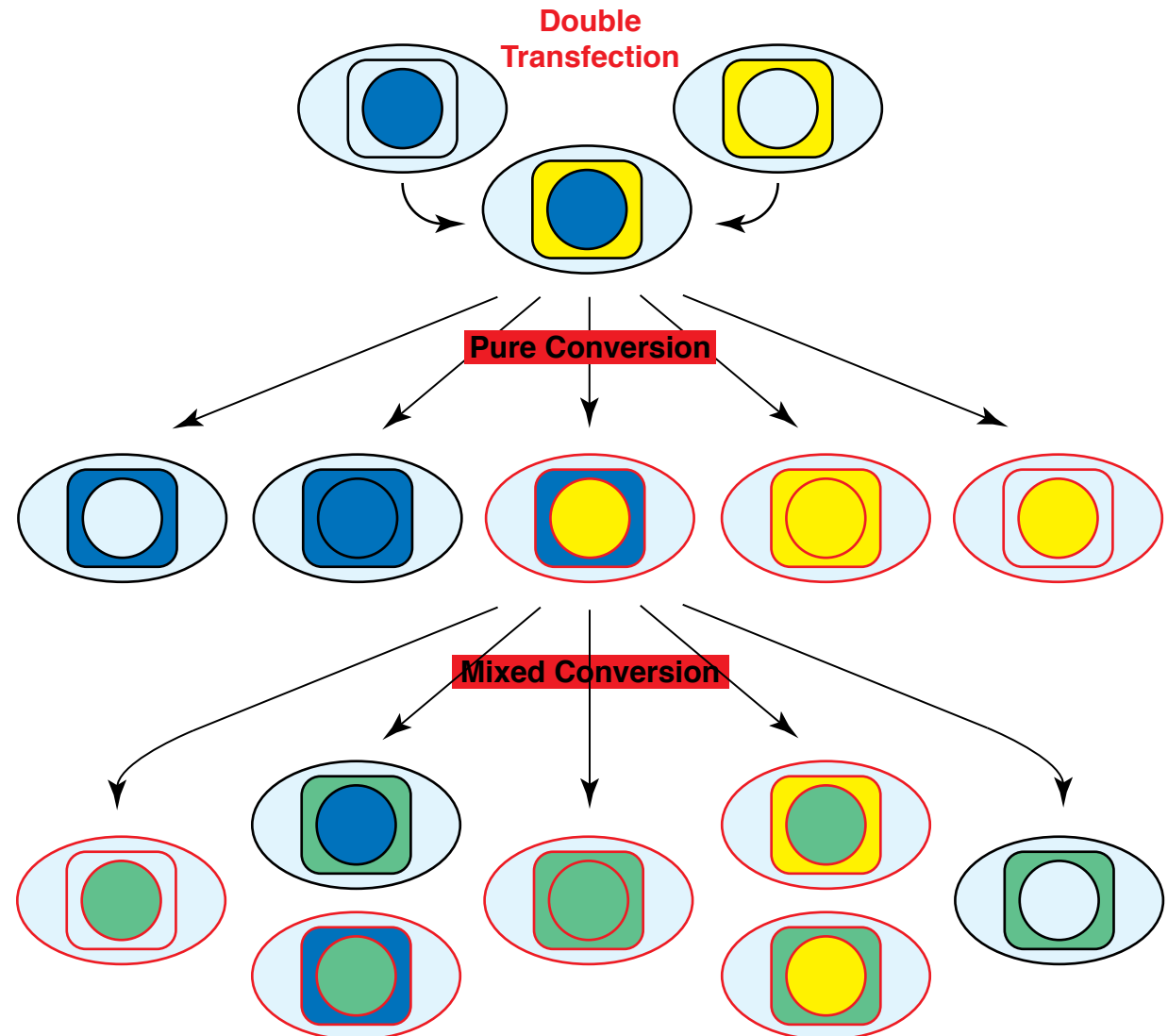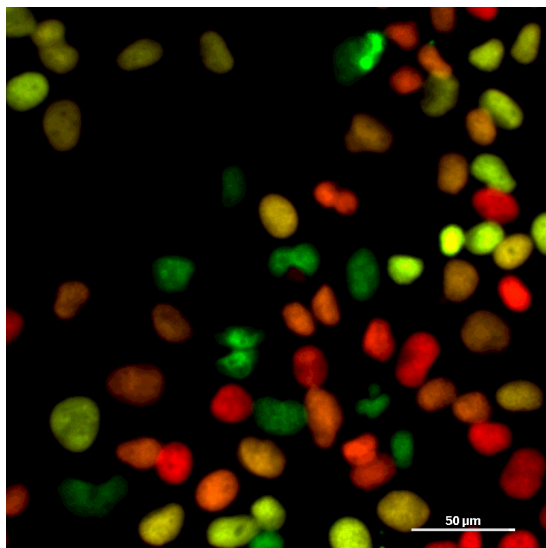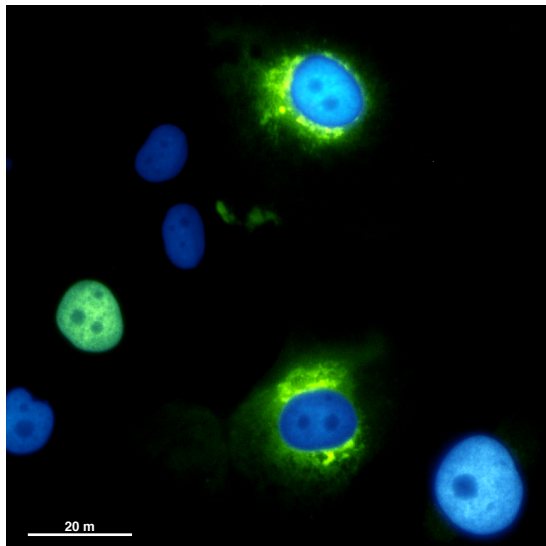
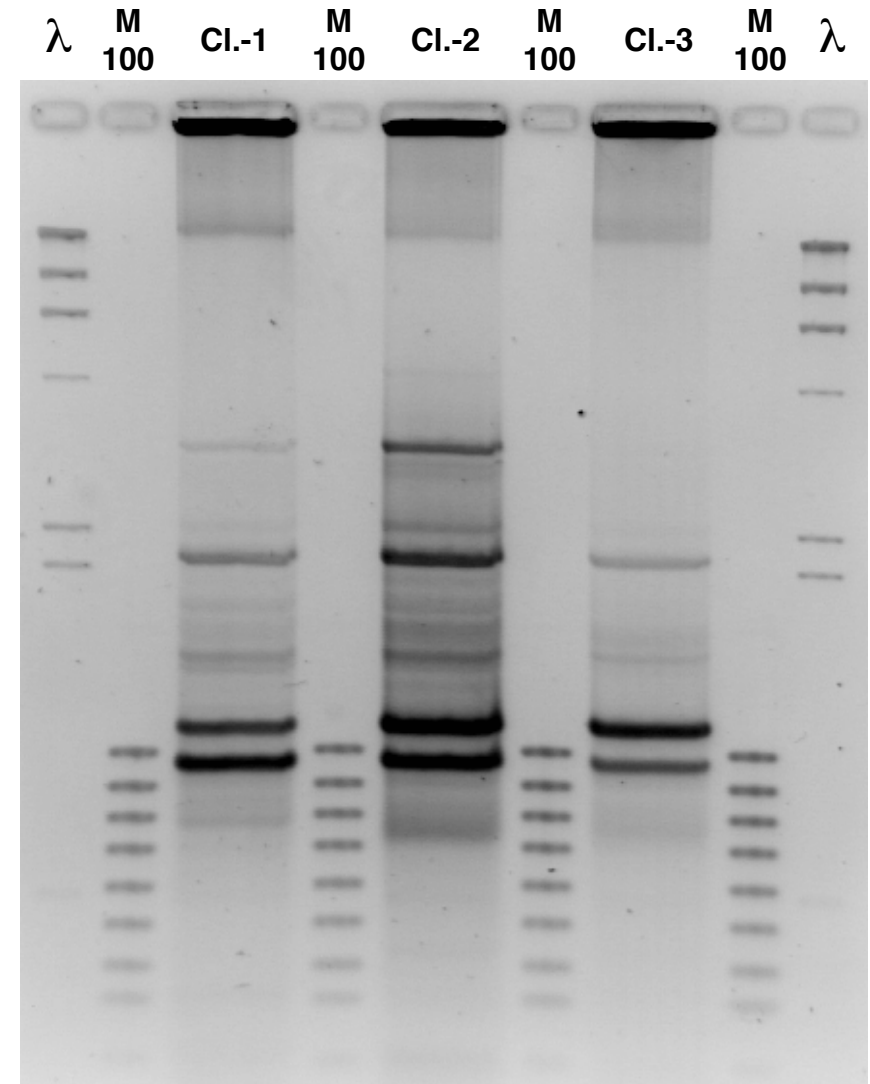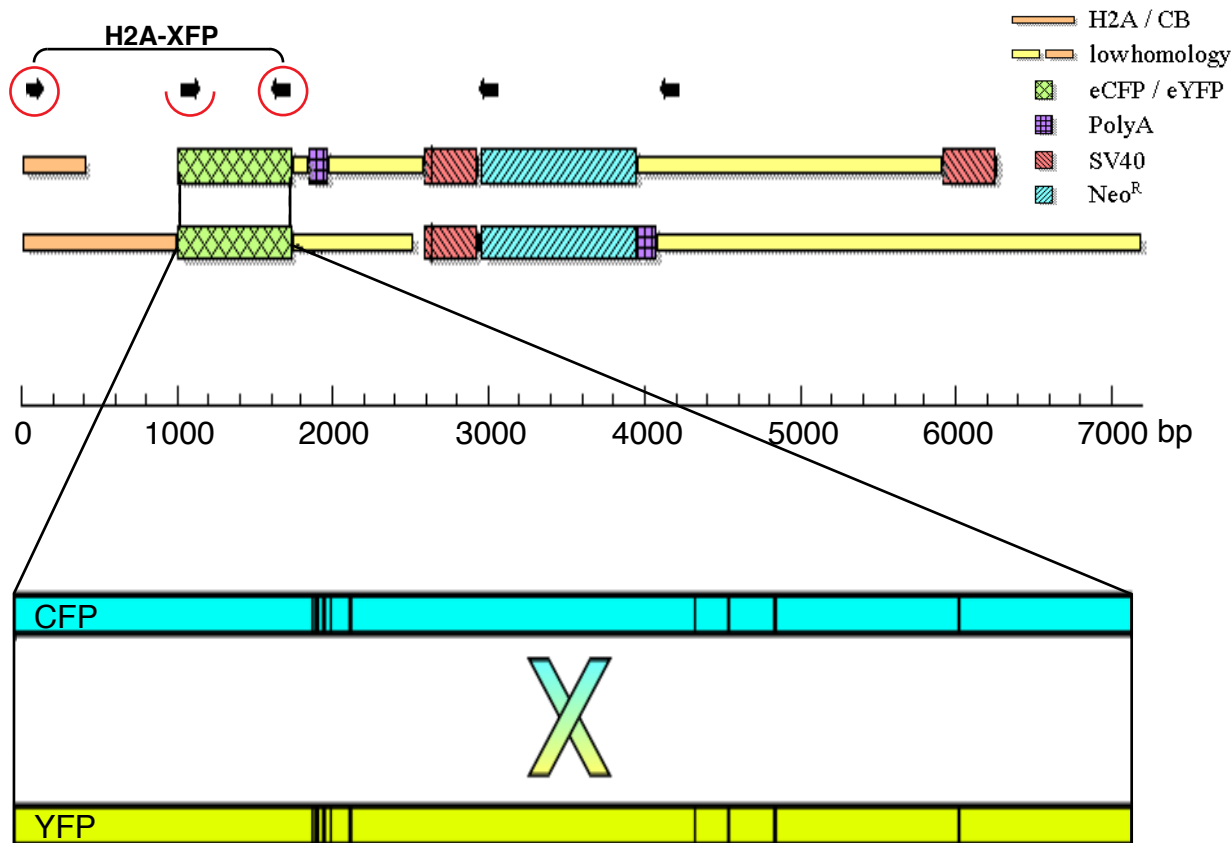# Homology analysis of vectors and genomic PCR for proof of conversion:
A homology analysis of H2A-CFP and CB-YFP suggests homologous recombination as cause of conversion.
The final proof of conversion was obtained with a genomic PCR of the full H2A-XFP fusion gene and sequencing of the PCR.
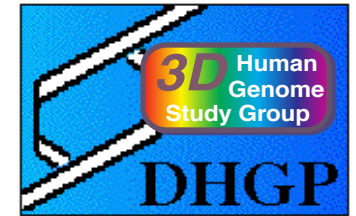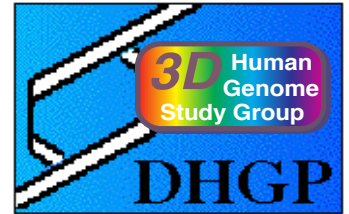
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Homology Comparison of H2A-CFP and CB-YFP

2% Agarose Gel, 40V, 4C, 20cm

Final proof of the conversion events by analysis of the sequenced PCR of an conversion enriched cell clone:
The conversion takes place in all 16 bp mutations separating CFP and YFP .
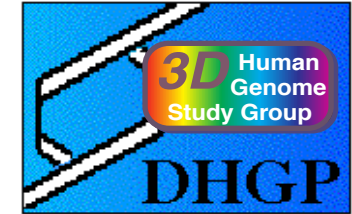
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H2A-ECFP       GGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACC
H2A-YFP (Cl.1) GGGATCCACCGGTCGCCACCATGGTGAGCAAGGGCGAGGAGCTGTTCACC
eYFP           -------------------ATGGTGAGCAAGGGCGAGGAGCTGTTCACC

H2A-ECFP       GGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAA
H2A-YFP (Cl.1) GGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAA
eYFP           GGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAA

H2A-ECFP       GTTCAGCGTGTCCGGCGAGGGCGAGGGCGA GCCACCTACGGCAAGCTGA
H2A-YFP (Cl.1) GTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGA
eYFP           GTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGA

H2A-ECFP       CCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC
H2A-YFP (Cl.1) CCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC
eYFP           CCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCCTGGCCCACC

H2A-ECFP       CTCGTGACCACCCTGACCTGGGGCGTGCAGTGCTTCAGCCGCTACCCCGA
H2A-YFP (Cl.1) CTCGTGACCACCTTcggCTacGGCCTGCAGTGCTTCgcCCGCTACCCCGA
eYFP           CTCGTGACCACCTTcggCTacGGCCTGCAGTGCTTCgcCCGCTACCCCGA

H2A-ECFP       CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACG
H2A-YFP (Cl.1) CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACG
eYFP           CCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACG

H2A-ECFP       TCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGC
H2A-YFP (Cl.1) TCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGC
eYFP           TCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACAAGACCCGC

H2A-ECFP       GCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAA
H2A-YFP (Cl.1) GCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAA
eYFP           GCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAA

H2A-ECFP       GGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
H2A-YFP (Cl.1) GGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT
eYFP           GGGCATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGT

H2A-ECFP       ACAACTACATCAGCCACAACGTCTATATCACCGCCGACAAGCAGAAGAAC
H2A-YFP (Cl.1) ACAACTACAaCAGCCACAACGTCTATATCAtgGCCGACAAGCAGAAGAAC
eYFP           ACAACTACAaCAGCCACAACGTCTATATCAtgGCCGACAAGCAGAAGAAC

H2A-ECFP       GGCATCAAGGCCAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGT
H2A-YFP (Cl.1) GGCATCAAGGtgAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGT
eYFP           GGCATCAAGGtgAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGT

H2A-ECFP       GCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG
H2A-YFP (Cl.1) GCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG
eYFP           GCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCG

H2A-ECFP       TGCTGCTGCCCGACAACCACTACCTGAGCACCAGTCCGCCCTGAGCAAA
H2A-YFP (Cl.1) TGCTGCTGCCCGACAACCACTACCTGAGCtaCCAGTCCGCCCTGAGCAAA
eYFP           TGCTGCTGCCCGACAACCACTACCTGAGCtaCCAGTCCGCCCTGAGCAAA

H2A-ECFP       GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGC
H2A-YFP (Cl.1) GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGC
eYFP           GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGC

H2A-ECFP       CGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA
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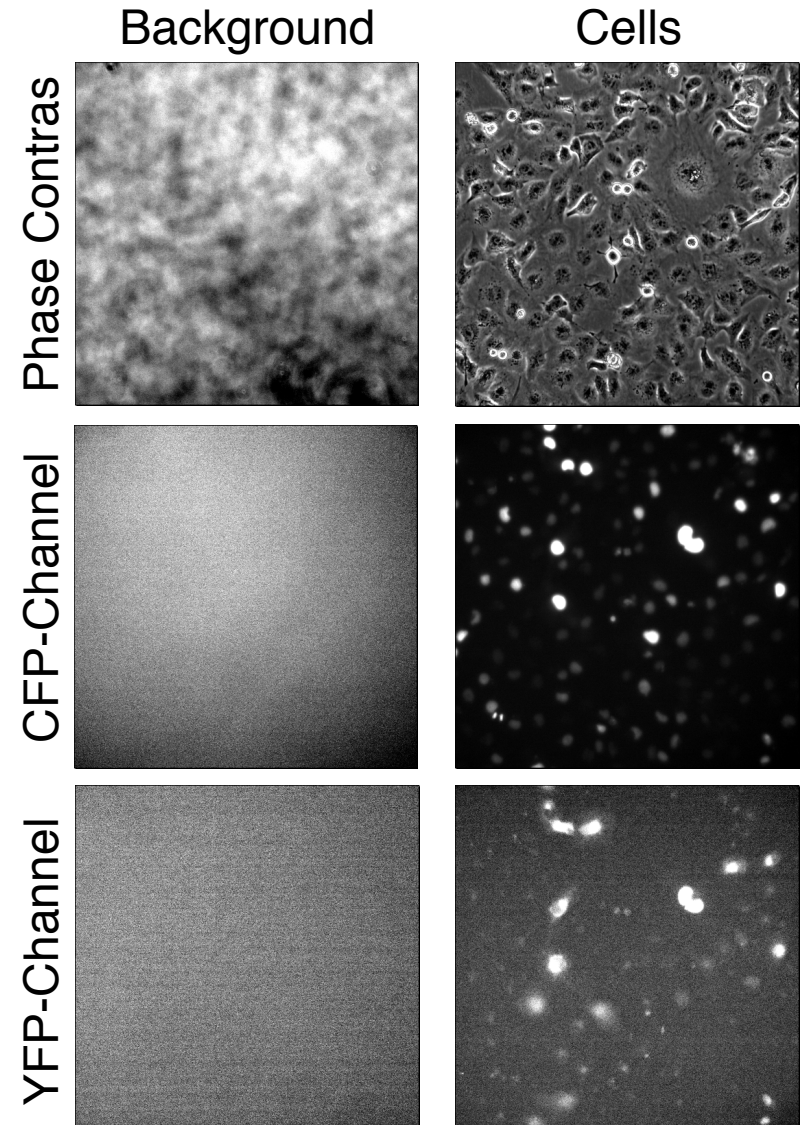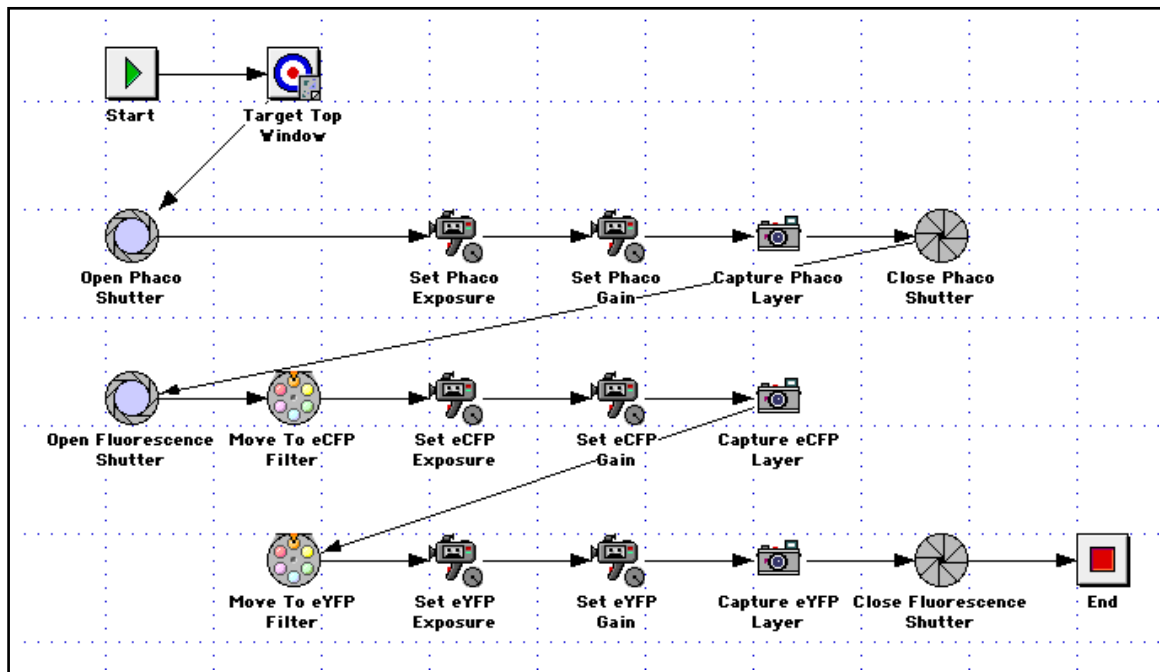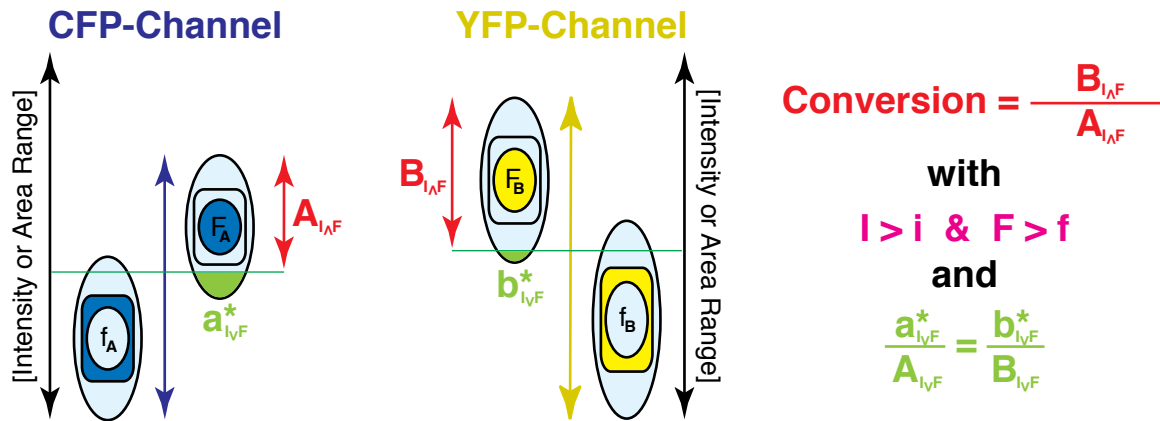
Quantifiing the conversion rate:
For quantifiing the conversion rate images were taken in the phase contrast-, CFP- and YFP- channel with a Zeiss Axiovert S100 TV. The image aquisition can partly be automized with macros.
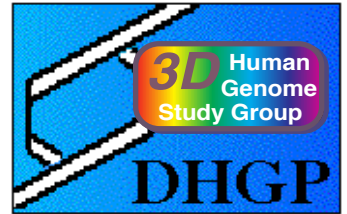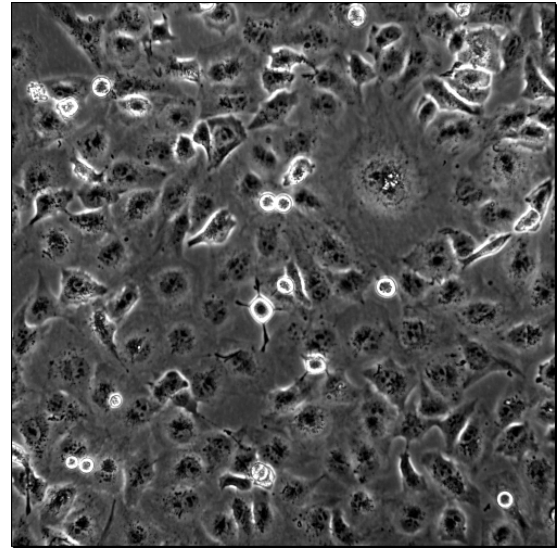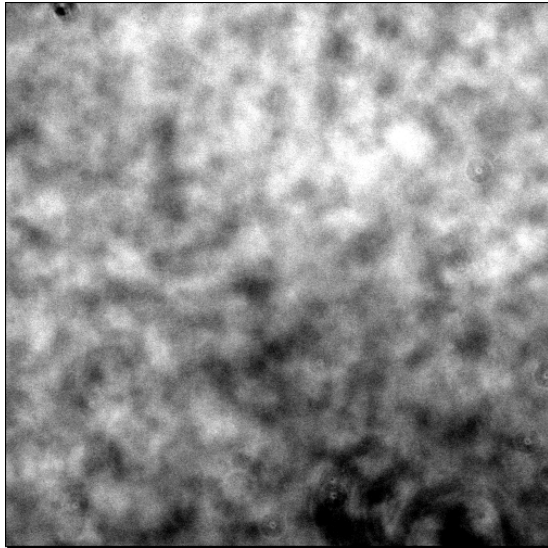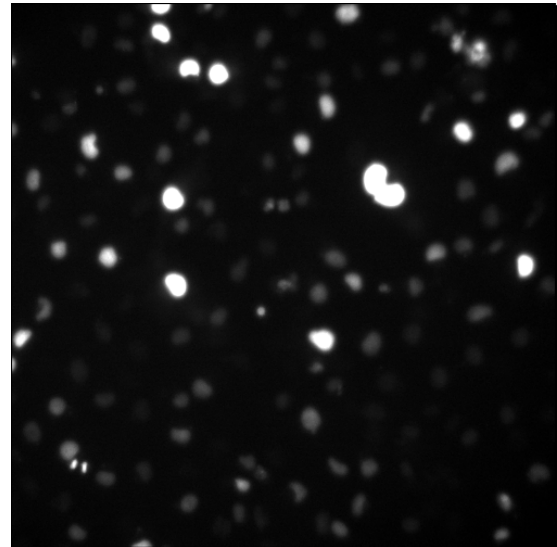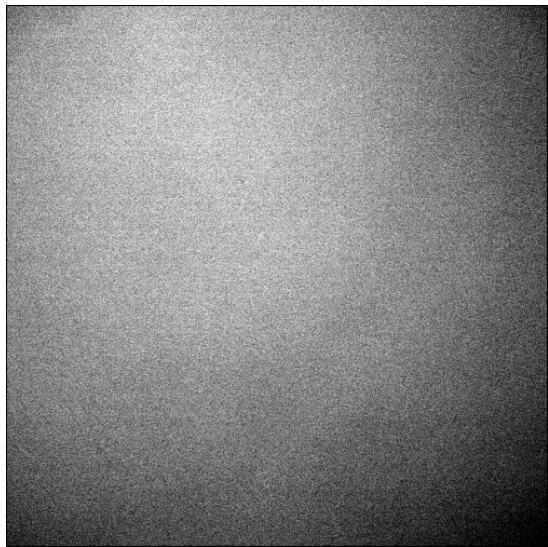Reliable conversion rates critically depend on many a parameter!

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Quantifiing the conversion - Image Aquisition:

For quantifiing the conversion rate images were taken in the phase contrast-, CFP- and YFP- channel with a Zeiss Axiovert S100 TV.
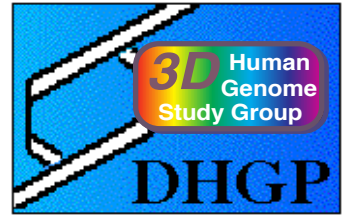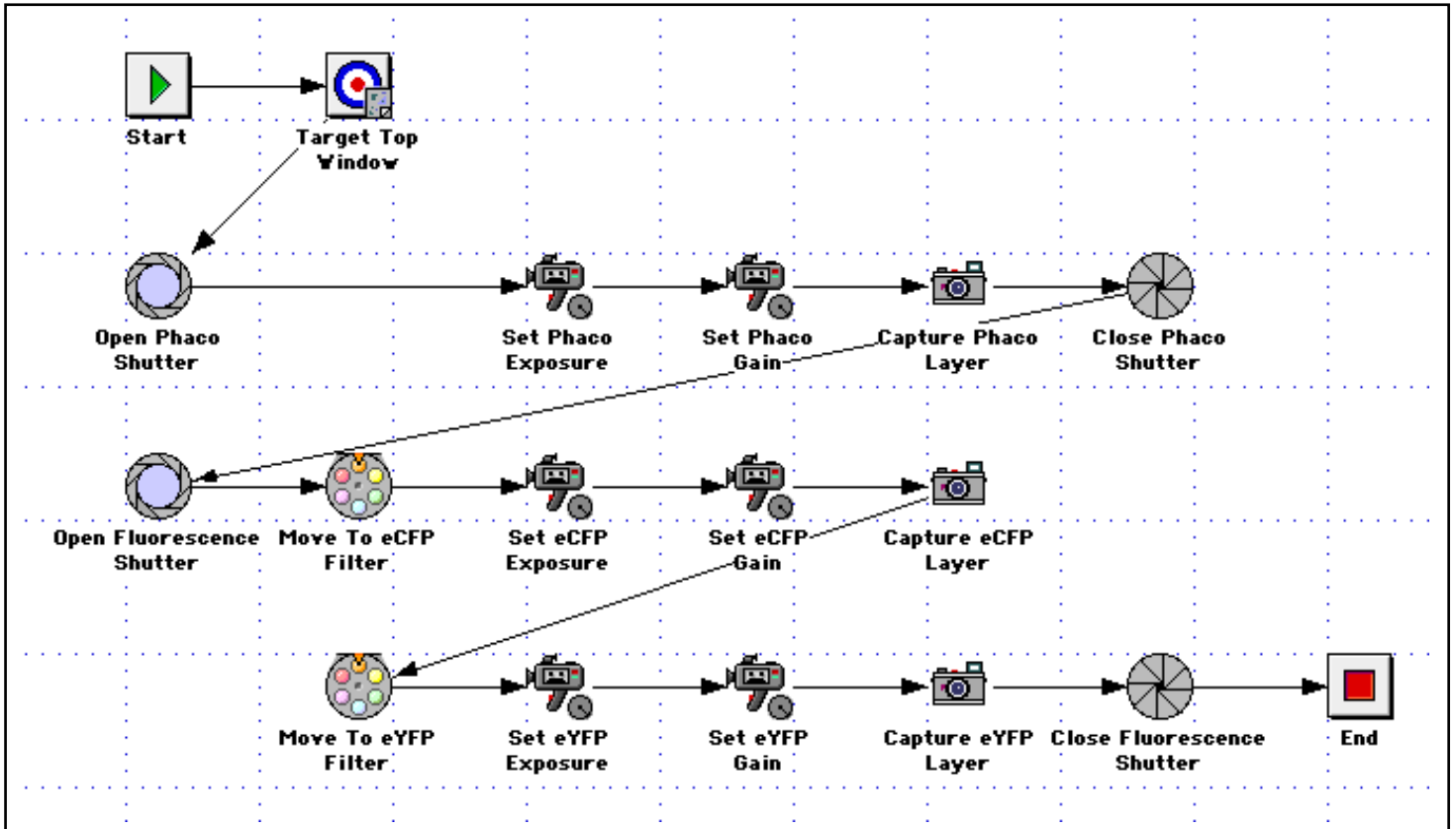
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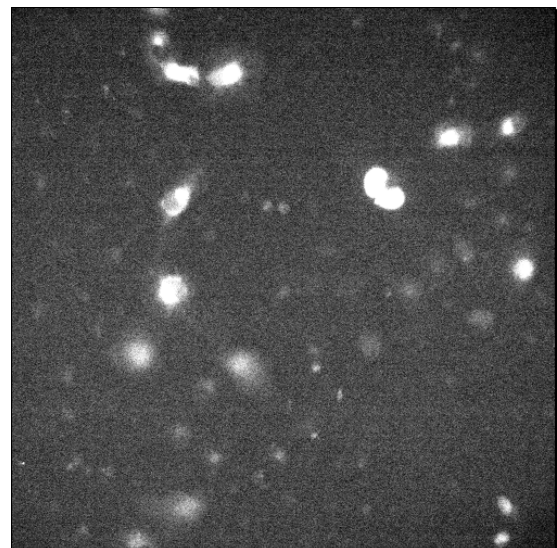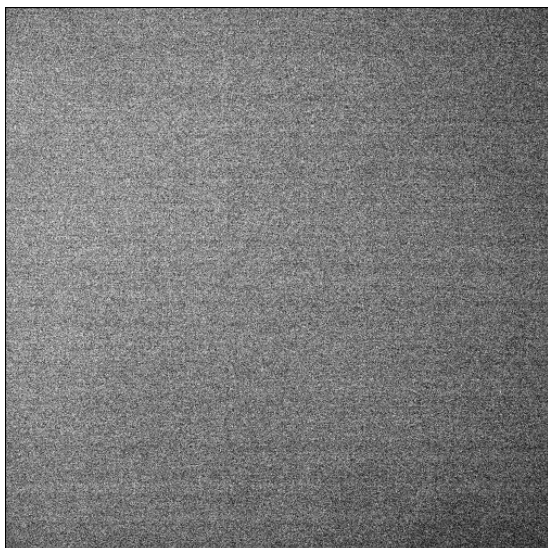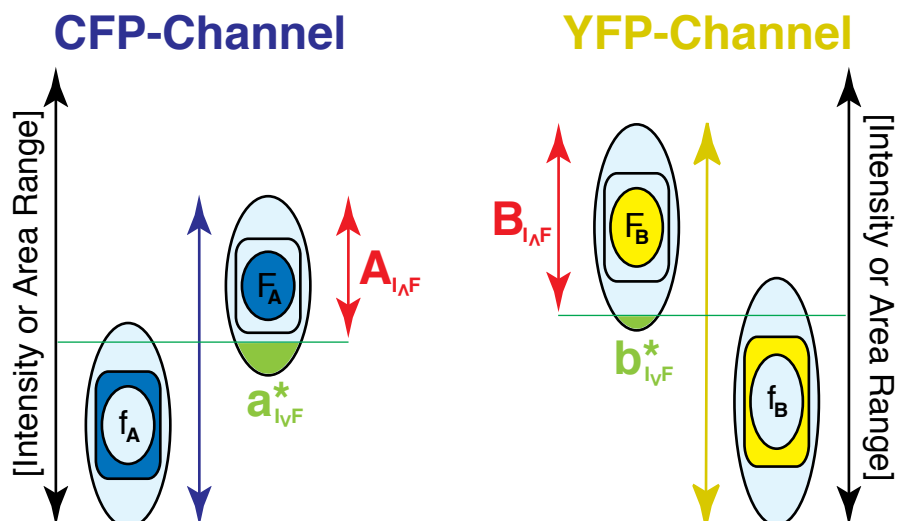
Quantifiing the conversion - Image Aquisition:
Although the image taking can partly be automized by macros, a reliable conversion rate depends critically on the aquisition parameters as well as on the vector system !

3D Human Genome Study Group

DHGP

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Start → **Target Top Window**

Open Phaco Shutter → **Set Phaco Exposure** → **Set Phaco Gain** → **Capture Phaco Layer** → **Close Phaco Shutter**

Open Fluorescence Shutter → **Move To eCFP Filter** → **Set eCFP Exposure** → **Set eCFP Gain** → **Capture eCFP Layer**

Move To eYFP Filter → **Set eYFP Exposure** → **Set eYFP Gain** → **Capture eYFP Layer** → **Close Fluorescence Shutter** → **End**

$$\text{Conversion} = \frac{B_{I \wedge F}}{A_{I \wedge F}} \quad \text{with} \quad I > i \ \& \ F > f \quad \text{and} \quad \frac{a^*_{I \vee F}}{A_{I \vee F}} = \frac{b^*_{I \vee F}}{B_{I \vee F}}$$

CFP-Channel

[Intensity or Area Range]

F_A $A_{I \wedge F}$

$a^*_{I \vee F}$

f_A

YFP-Channel

$B_{I \wedge F}$ F_B

$b^*_{I \vee F}$

f_B
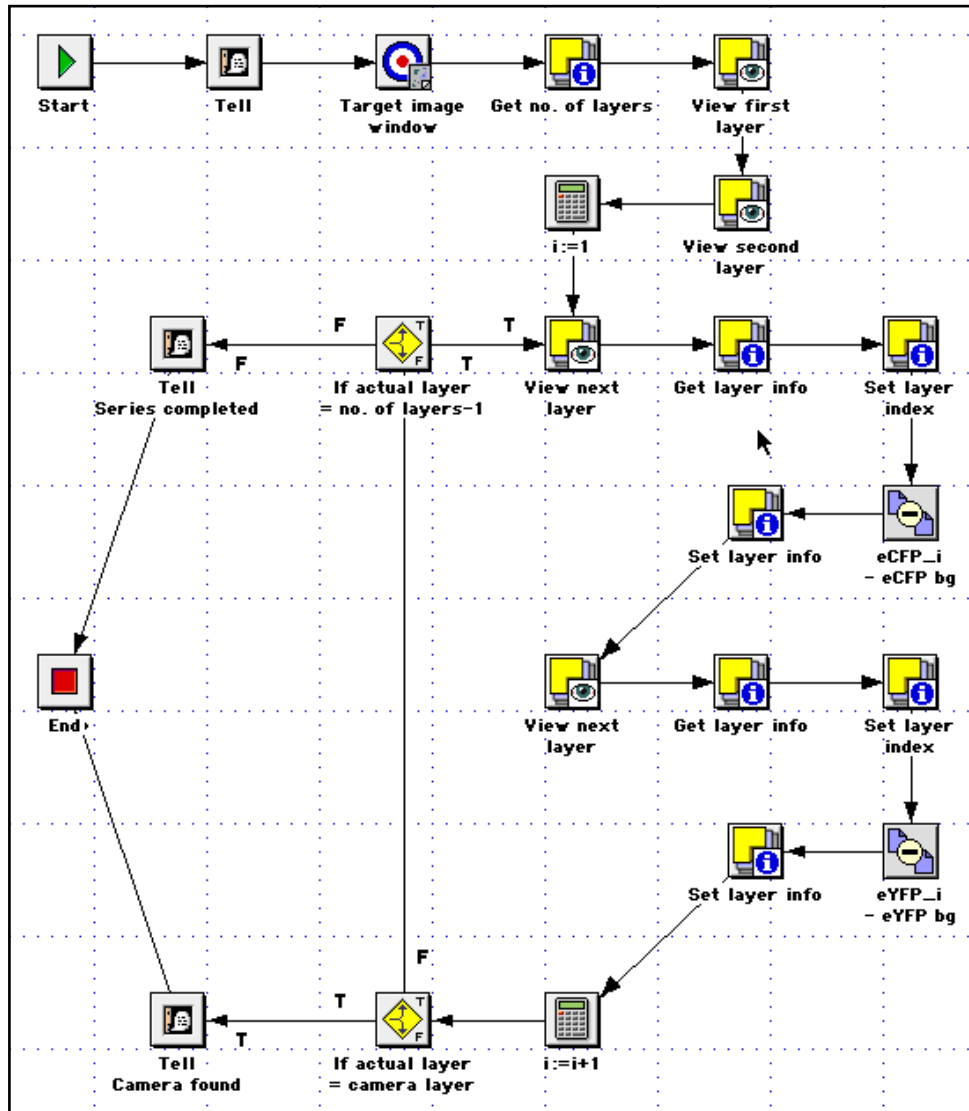
[Intensity or Area Range]

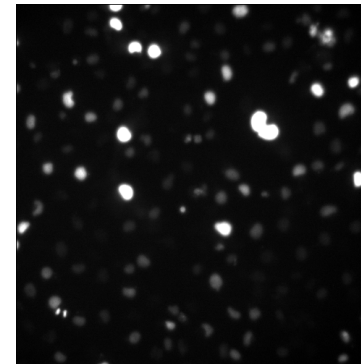# Quantifiing the conversion - Numeration and Background Subtraction:

For later digital image processing the images have to be numbered and the background having no isotrope distribution accross the field of illumination is subtracted using again a macro.
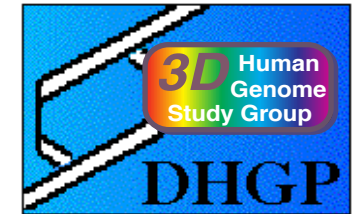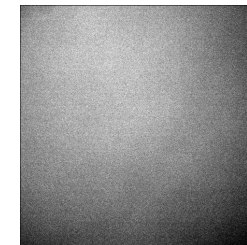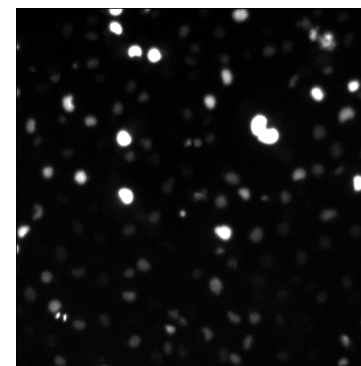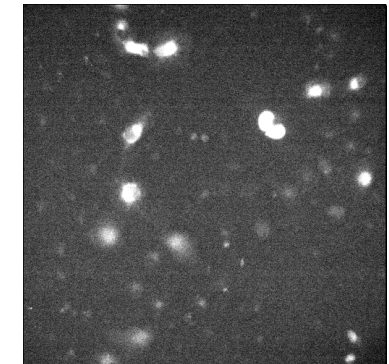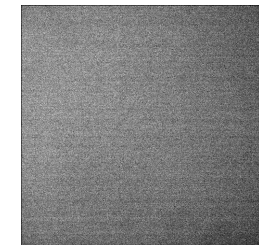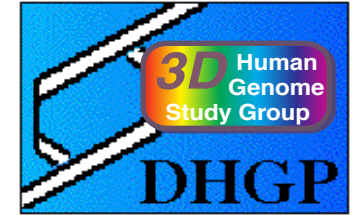
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CFP-Channel

YFP-Channel
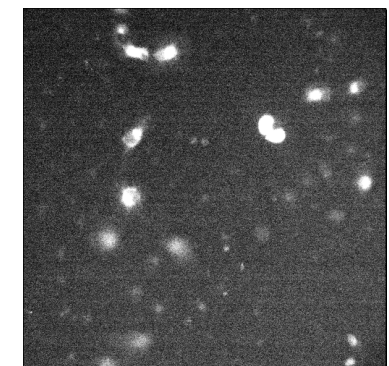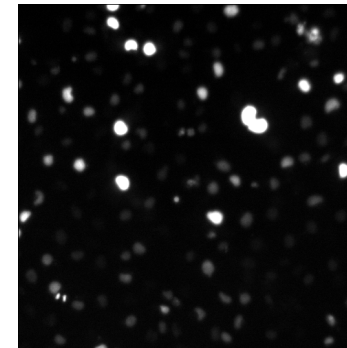
Quantifiing the conversion - Signal Analysis:
With intensity thresholds, binary masks of each channel are created separating most H2A from CB signals. With the merged binary masks the signals in each channel are reevaluated, followed by an area separation of the H2A from the CB signal. The data can be checked in a result image.
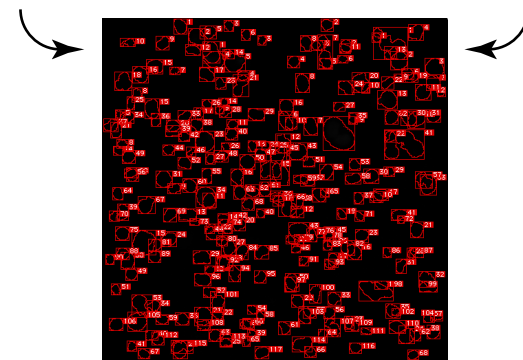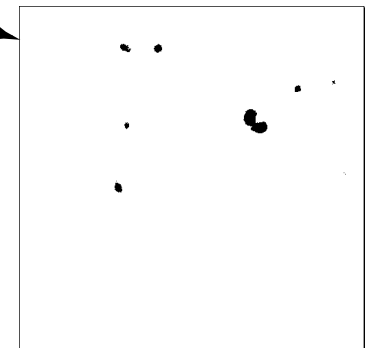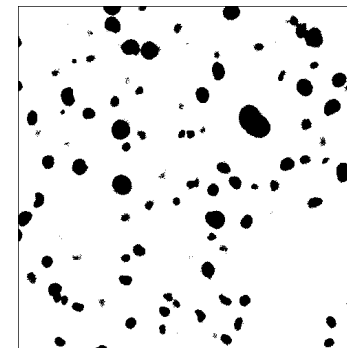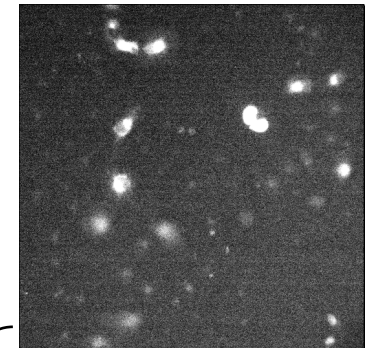
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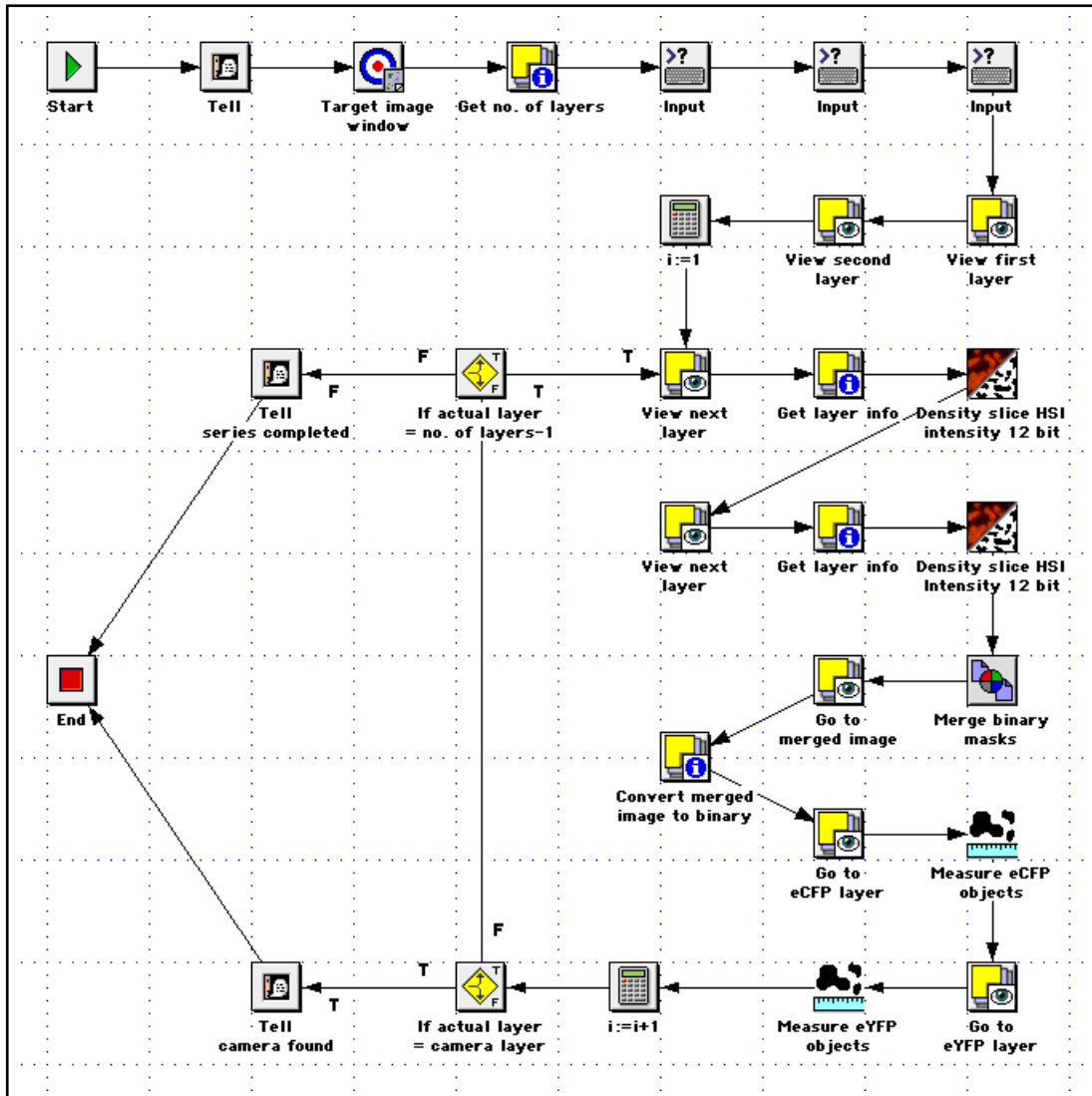
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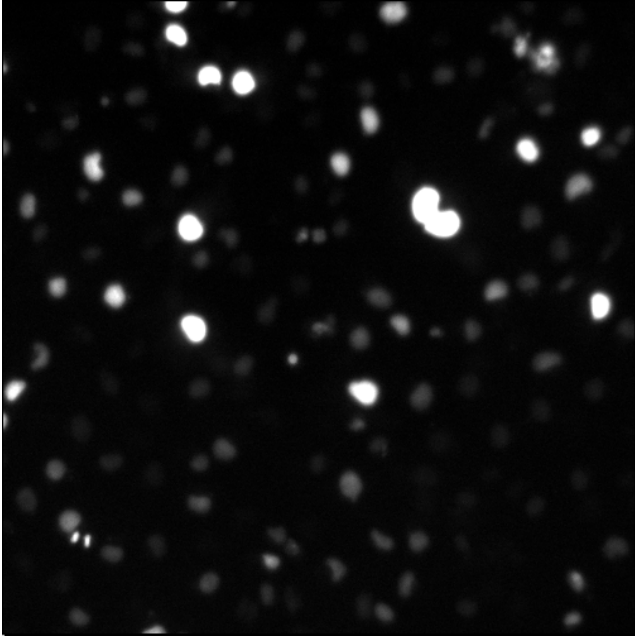
YFP-Channel
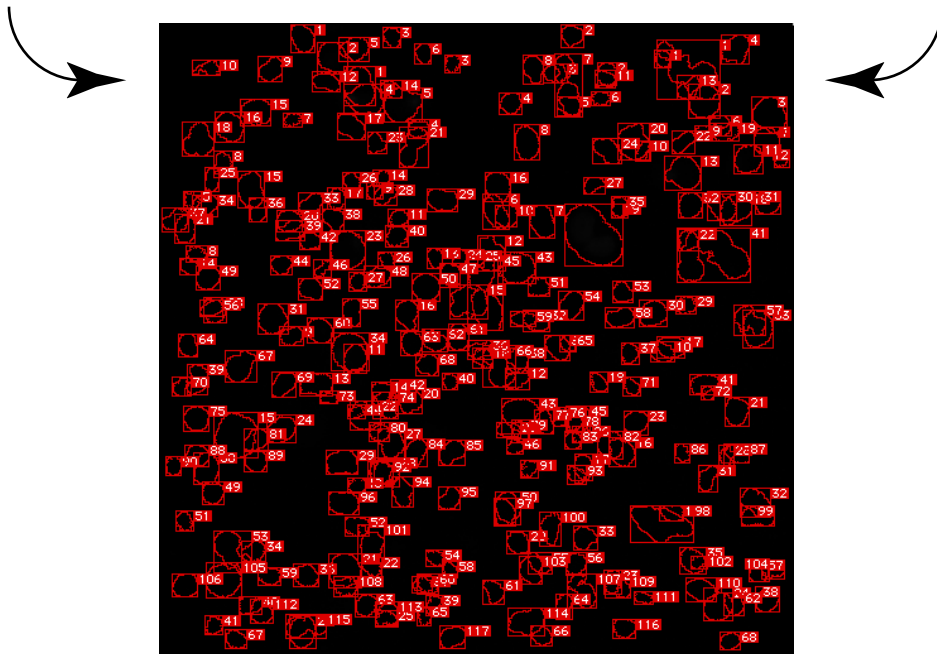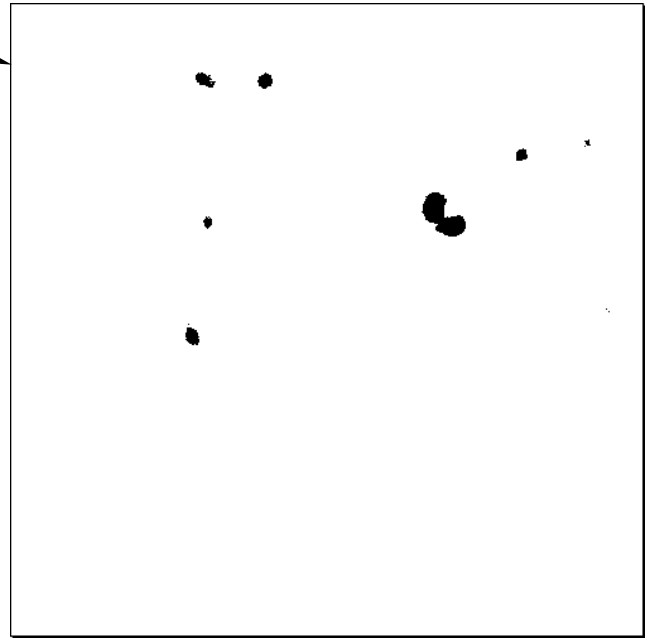
Various conversion experiments proof the high rate of conversion:
For sufficient statistics in each experiment 1000 to 3000 signals were obtained, including tests totaling to more than 50.000.
In general conversion appears with different vectors, cell lines, and methods of transfection, but not in overtransfection of a stable cell line !
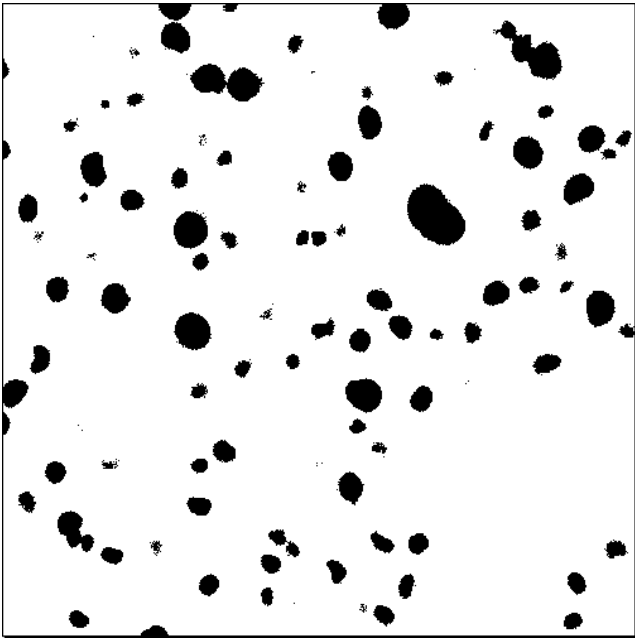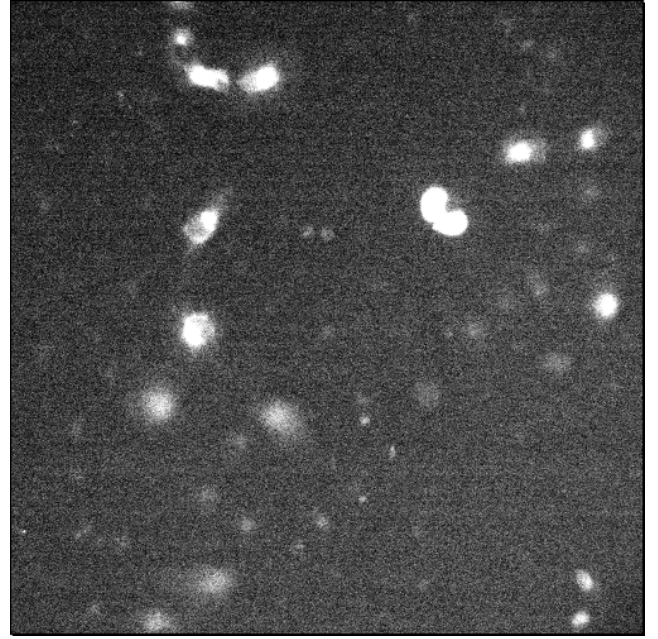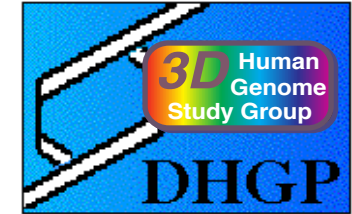
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Construct	Cells	Method		Conversion [+/-] and [%]	
H2A-CFP	LCLC103H	FuGene6		-	0.0
CB-YFP	LCLC103H	FuGene6		-	0.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	simultanious	+	4.0 0.2
H2A-CFP + DsRed	LCLC103H	FuGene6	simultanious	-	0.0
H2A-CFP + pure GFP	LCLC103H	FuGene6	simultanious	+	>1.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	sep. Mix + simultanious	+	>1.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	sep. Mix + 4 h delay	- (+)	0.0 (7.3)
H2A-CFP* + CB-YFP	LCLC103H	FuGene6	overtransfec. *stable line	-	0.0
H2A-CFP + CB-YFP	LCLC103H	FuGene6	5x DNA conc	+	?
H2A-CFP + CB-YFP	LCLC103H	FuGene6	10x DNA conc	+	?
H2A-CFP + CB-YFP	LCLC103H	FuGene6	linearized	+	3.4
H2A-CFP + CB-YFP	LCLC103H	FuGene6	linearized + 96C	+++	10.3
H2A-CFP + CB-YFP	LCLC103H	Dmrie-C	simultanious	+	3.8
H2A-CFP + CB-YFP	LCLC103H	Cellfectin	simultanious	+	>1.0
H2A-CFP + CB-YFP	LCLC103H	Lipofectin	simultanious	+	2.4
H2A-CFP + CB-YFP	LCLC103H	GibcoPlus	simultanious	+	>1.0
H2A-CFP + CB-YFP	LCLC103H	Electroporation	simultanious	+	~1.0
H2A-CFP + CB-YFP	LCLC103H	Ca-Phosphat	simultanious	+	~1.0
H2A-CFP	HeLa	FuGene6		-	0.0
H2A-CFP + CB-YFP	HeLa	FuGene6	simultanious	+	?
H2A-CFP	Cos-7	FuGene6		-	0.0
H2A-CFP + CB-YFP	Cos-7	FuGene6	simultanious	+	?

Summary and Outlook

Tobias A. Knoch

Warning

simultanious cotransfections can lead to GFP-walking

**the artificial and misleading results due to conversion
are usually between 2% and 8%
and can reach up to ~20% in the extreem**

**the conversion can be reduced dramatically
by succesive transfection
and overtransfection of a stably transfected cell line**

Application

**a fast method for expressing a fusion protein with
another GFP without creating a new DNA plasmid
and receiving immediately a clone of a cell line**

**this system is a suitable tool for the investigation
of homologous recombination
(e.g. cancer cells with elevated recombination activity)
or similar processes
(an optimized system for FACS analysis is possible)**

CFP-Channel
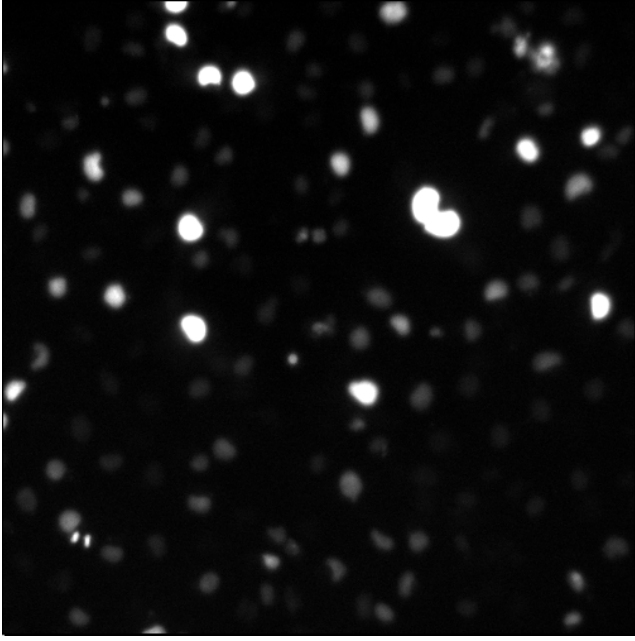
YFP-Channel
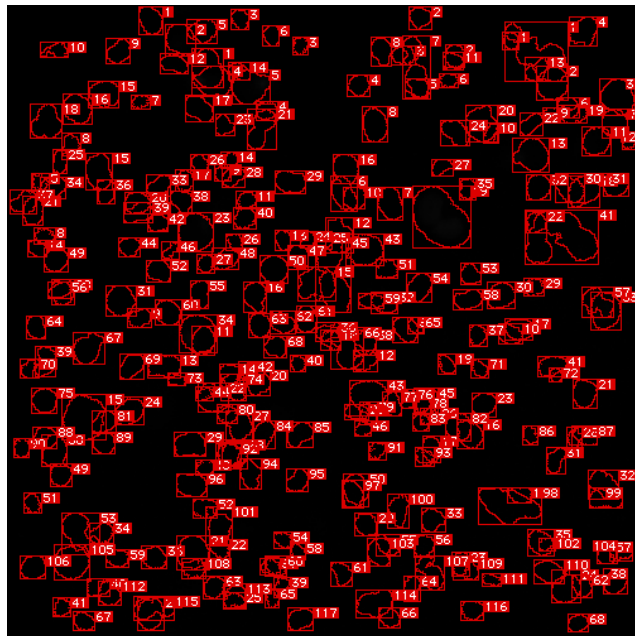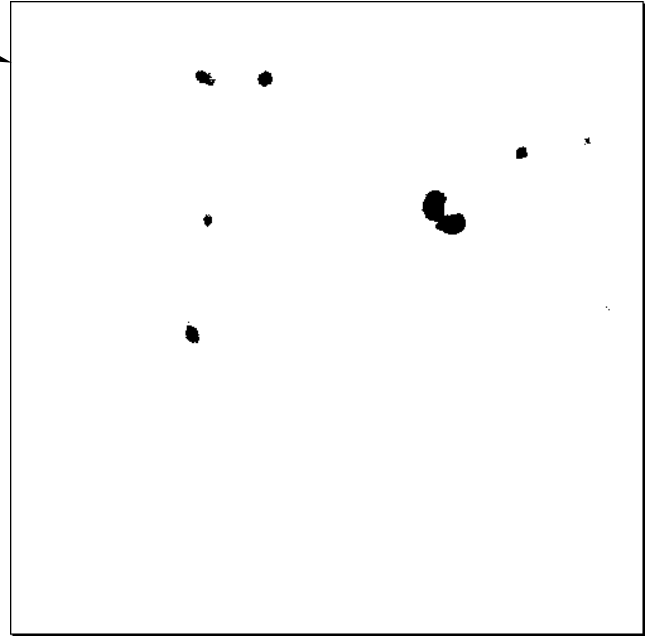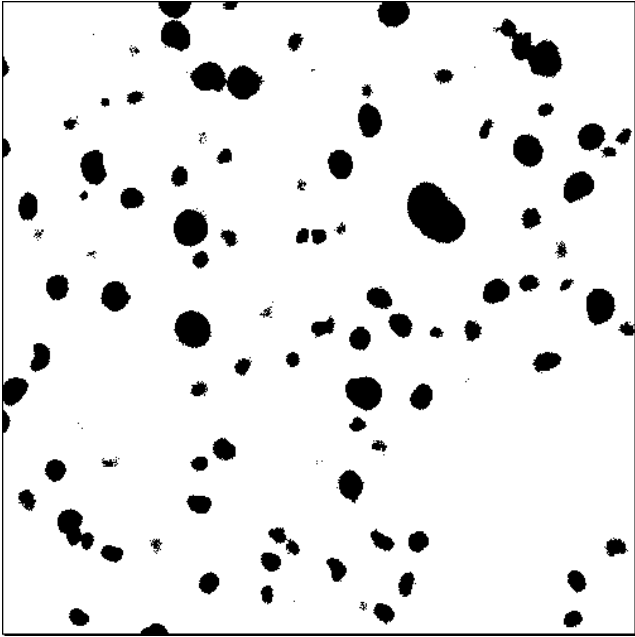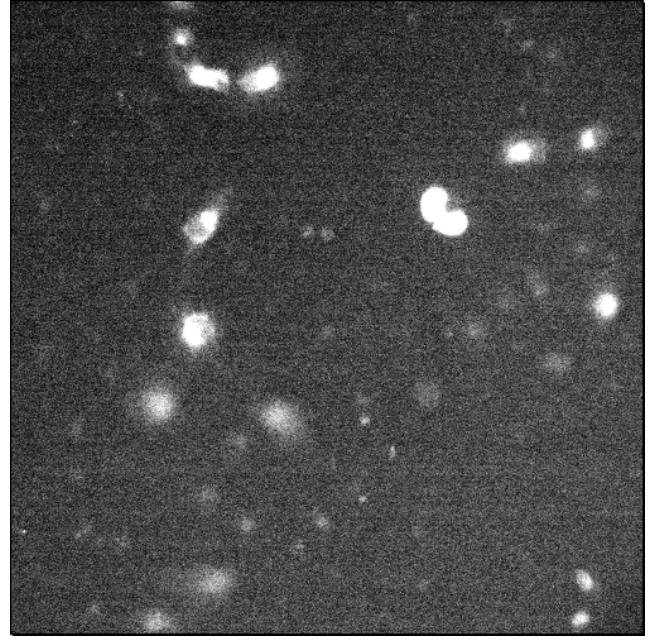
"GFP-Walking": Artificial Construct Aberrations caused by Co-Transfectional Homologous Recombination

Knoch, T. A.

Biophysics of Macromolecules Seminar, German Cancer Research Centre (DKFZ),
Heidelberg, Germany, July, 2000.

Corresponding author email contact: TA.Knoch@taknoch.org

Literature References

Knoch, T. A. Dreidimensionale Organisation von Chromosomen-Domänen in Simulation und Experiment. (Three-dimensional organization of chromosome domains in simulation and experiment.) *Diploma Thesis*, Faculty for Physics and Astronomy, Ruperto-Carola University, Heidelberg, Germany, 1998, and TAK Press, Tobias A. Knoch, Mannheim, Germany, ISBN 3-00-010685-5 and ISBN 978-3-00-010685-9 (soft cover, 2rd ed.), ISBN 3-00-035857-9 and ISBN 978-3-00-0358857-0 (hard cover, 2rd ed.), ISBN 3-00-035858-7, and ISBN 978-3-00-035858-6 (DVD, 2rd ed.), 1998.

Knoch, T. A., Münkel, C. & Langowski, J. Three-dimensional organization of chromosome territories and the human cell nucleus - about the structure of a self replicating nano fabrication site. *Foresight Institute - Article Archive*, Foresight Institute, Palo Alto, *CA, USA,* http://www. foresight.org, 1- 6, 1998.

Knoch, T. A., Münkel, C. & Langowski, J. Three-Dimensional Organization of Chromosome Territories and the Human Interphase Nucleus. *High Performance Scientific Supercomputing*, editor Wilfried Juling, Scientific Supercomputing Center (SSC) Karlsruhe, University of Karlsruhe (TH), 27- 29, 1999.

Knoch, T. A., Münkel, C. & Langowski, J. Three-dimensional organization of chromosome territories in the human interphase nucleus. *High Performance Computing in Science and Engineering 1999*, editors Krause, E. & Jäger, W., High-Performance Computing Center (HLRS) Stuttgart, University of Stuttgart, Springer Berlin-Heidelberg-New York, ISBN 3-540-66504-8, 229-238, 2000.

Bestvater, F., **Knoch, T. A.**, Langowski, J. & Spiess, E. GFP-Walking: Artificial construct conversions caused by simultaneous cotransfection. *BioTechniques* 32(4), 844-854, 2002.