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Supplemental Information

FRET Image Correlation Spectroscopy Reveals RNAPII-Independent P-TEFb Recruitment on Chromatin

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TABLE S1

	5'-forward-3'	5'-reverse-3'
Cloning		
CT1*wt*-VLL-XFP-N	GATCCCGCGGTGAGGGAGAGAGGAAGAACAACAA	AGTCGCGGCCGCCTACTTAGGAAGGGGTGGAAGTGG
CT1*wt*-VLL-XFP-C	GATCGCTAGCCGCCACCATGGAGGGAGAGAGGAAGAACAACA	GATCACCGGTCTTAGGAAGGGGTGGAAGTGGTG
CT1*wt*VLL-XFP-N	GATCCCGCGGTGGTCCAAAAAAGAAGAGAAAGGTAGGA	AGTCGCGGCCGCCTACTTAGGAAGGGGTGGAAGTGG
CT1*wt*-VLL-XFP-C	GATCGCTAGCCGCCACCATGGGTCCAAAAAAGAAGAGAAAGGTAGGATCCAT	GATCACCGGTCTTAGGAAGGGGTGGAAGTGGTG
RPB1-VLL-XFP-C	GATCGCTAGCCGCCACCATGCACGGGGGTGGCCC	GATCACCGGTTCTAGCGTTCTCCTCGTCACTGTC
RPB1-VLL-XFP-N	GATCCCGCGGTCACGGGGGTGGCCCC	AGTCGCGGCCGCTTATCTAGCGTTCTCCTCGTCACTGTC

Table S1: Oligonucleotides used for gene or cDNA cloning.

SUPPLEMENTAL FIGURE LEGENDS.

FIGURE S1. *Optimization of FRET fluorescent pair and structure of fluorescent chimeras.* **A,** The standardization of the process to build fluorescent fusion proteins and its rationalization to optimize FRET was achieved by the creation of a directed cloning site instead of the standard multicloning site of the peGFP-N vector. This directed cloning site includes a 5' pair of restriction sites (Nhe I / Age I) and a 3' pair of restriction sites (Sac II / Not I) which surround a linker sequence. This linker encodes a 42 AA peptide which can be reduced to either 22 AA or 12 AA by a single restriction of the vector with BstE II or SgrA I, respectively. This vector is dubbed variable length linker plasmid (pVLL). **B,** Chart summarizing results of frequency-domain FLIM experiments performed in U2OS cells transfected with pVLL vectors expressing either mT2, CT1-mT2 or RPB1-SYFP2 linked at the N or C terminal part. The effect of the three different lengths of VLL on FRET efficiency was also evaluated. Controls with non-fused pairs are presented and mT2 alone as well. a, b, c, d: significance reached ($p<0.05$) with the respective control (grey bars). Values represent Mean phase lifetime (τ_φ) ± SD. **C,** Comparison of FRET efficiency of both fluorescent pairs: mTurquoise2 / SYFP2 and mTurquoise2 / Reach2 as determined by FLIM-FRET in U2OS cells. Fluorescent pairs expressed concomitantly (i.e. mTurquoise2 + SYFP2) have been compared with fluorescent pairs fused in frame in the pVLL vector (i.e. SYFP2-VLL-mTurquoise2) and separated with linkers of different lengths (12, 22 or 42 amino acids). Fluorescence lifetime was assessed by frequency-domain FLIM imaging. Significant was considered for $p<0.05$; a: significantly different from the average lifetime distribution of mT2 expressing cells, b: significantly different from the average lifetime distribution of mT2 / SYFP2 homologue pair. **D**, Theoretical graph plot showing the relation between the FRET efficiency (E_{FRET}; %) and the distance between acceptor and donor of FRET. The Förster radius, R0, extracted from literature (15-18, 39) is calculated as explained in the method section. Among several acceptor-donor pairs, mTurquoise2 – Reach2 appears as the best performer.

FIGURE S2. *Variance in daily measurement of lifetime and between different chimeric donor of fluorescence.* **A**, Graph plot shows the residuals of CT1-mT2 lifetime in 25 independent experiments. **B**, Median values of the average lifetime of CT1*wt*, CT1Δ(503-533) or CT1Δ(480-551) from different experiments. Bars: mean±SD.

FIGURE S3. *Characterization and denoising of lifetime images.* **A,** Lifetime image of mT2 protein (top row) expressed in U2OS (left image) was filtered by a 1-pixel sliding binning along x/y directions

(middle image) or 2-pixel binning (right image). Extra-nuclear pixels were excluded from analysis (dark area). **B,** same as A for a cell expressing CT1-mT2 and H2A-Reach.

FIGURE S4. *Example of a dataset of simulated images generated to evaluate the error in calculated cluster surface area and cluster density.* 1 representative image over 20 is shown for each condition of cluster surface area and cluster density. Cluster shape: square.

FIGURE S5. *Evaluation of the correction of the calculated cluster density.* **A,** Graph plot showing the error in calculated cluster density as a function of particle density, with or without correction. **B,** Graph plot showing the residuals of the regression analysis of the two populations as a function of particle density, with or without correction.

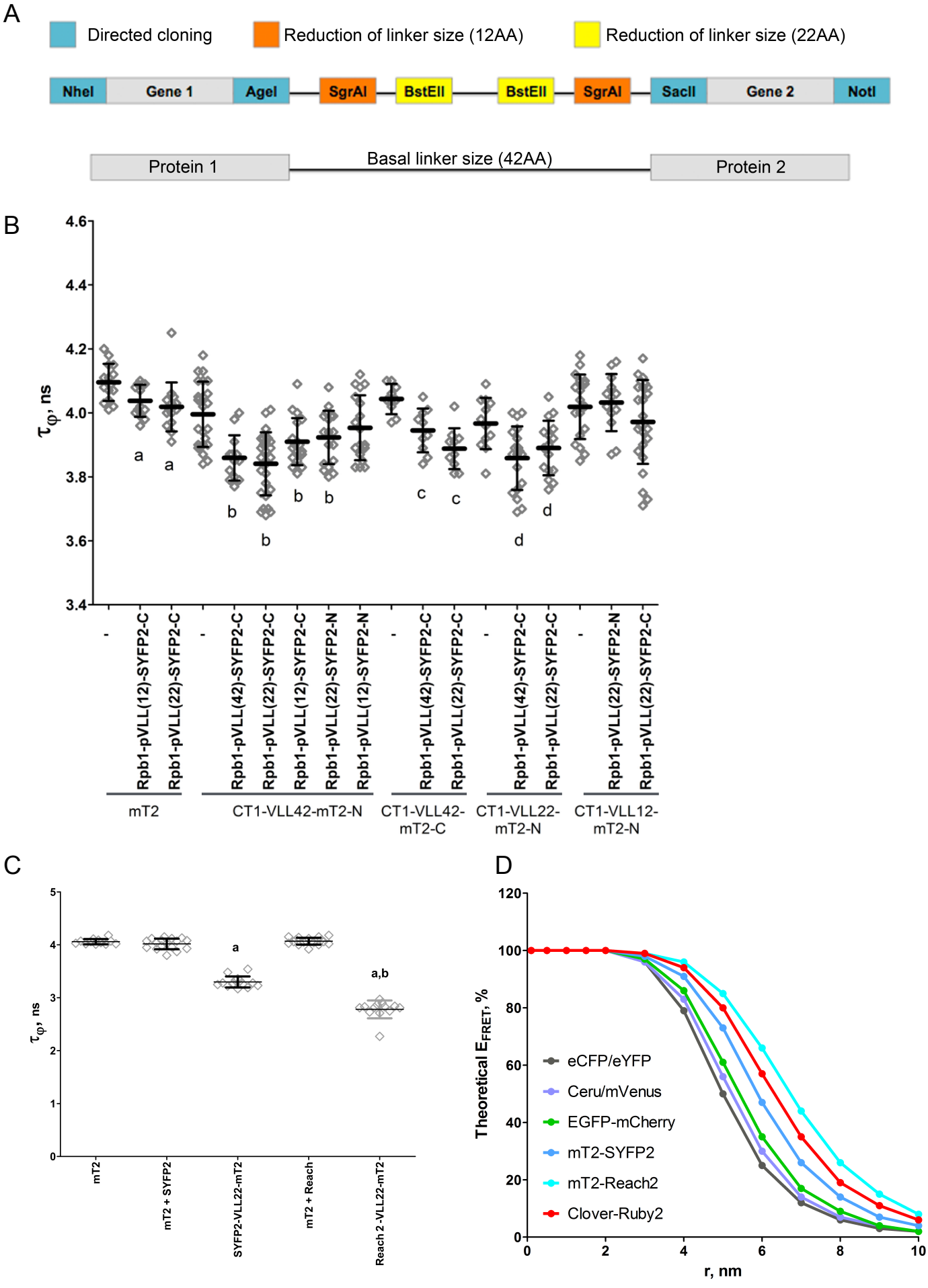
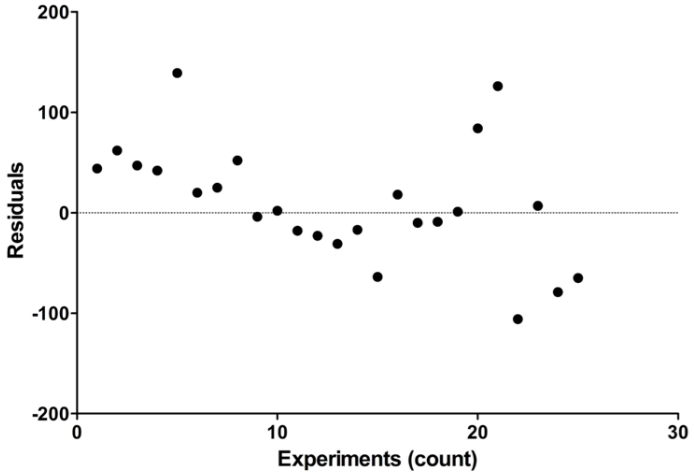

A
Directed cloning
Reduction of linker size (12AA)
Reduction of linker size (22AA)
NheI
Gene 1
AgeI
SgrAI
BstEII
BstEII
SgrAI
SacII
Gene 2
NotI
Protein 1
Basal linker size (42AA)
Protein 2
B
τφ, ns
4.6
4.4
4.2
4.0
3.8
3.6
3.4
a
a
b
b
b
b
c
c
d
d
-
Rpb1-pVLL(12)-SYFP2-C
Rpb1-pVLL(22)-SYFP2-C
Rpb1-pVLL(42)-SYFP2-C
Rpb1-pVLL(22)-SYFP2-C
Rpb1-pVLL(12)-SYFP2-C
Rpb1-pVLL(22)-SYFP2-N
Rpb1-pVLL(12)-SYFP2-N
Rpb1-pVLL(42)-SYFP2-C
Rpb1-pVLL(22)-SYFP2-C
Rpb1-pVLL(42)-SYFP2-C
Rpb1-pVLL(22)-SYFP2-C
Rpb1-pVLL(22)-SYFP2-N
Rpb1-pVLL(22)-SYFP2-C
mT2
CT1-VLL42-mT2-N
CT1-VLL42-mT2-C
CT1-VLL22-mT2-N
CT1-VLL12-mT2-N
C
τφ, ns
5
4
3
2
1
0
a
a,b
mT2
mT2 + SYFP2
SYFP2-VLL22-mT2
mT2 + Reach
Reach 2-VLL22-mT2
D
Theoretical EFRET, %
120
100
80
60
40
20
0
0
2
4
6
8
10
r, nm
eCFP/eYFP
Ceru/mVenus
EGFP-mCherry
mT2-SYFP2
mT2-Reach2
Clover-Ruby2

Fig S1

A


200
100
0
-100
-200
Residuals
0
10
20
30
Experiments (count)

B

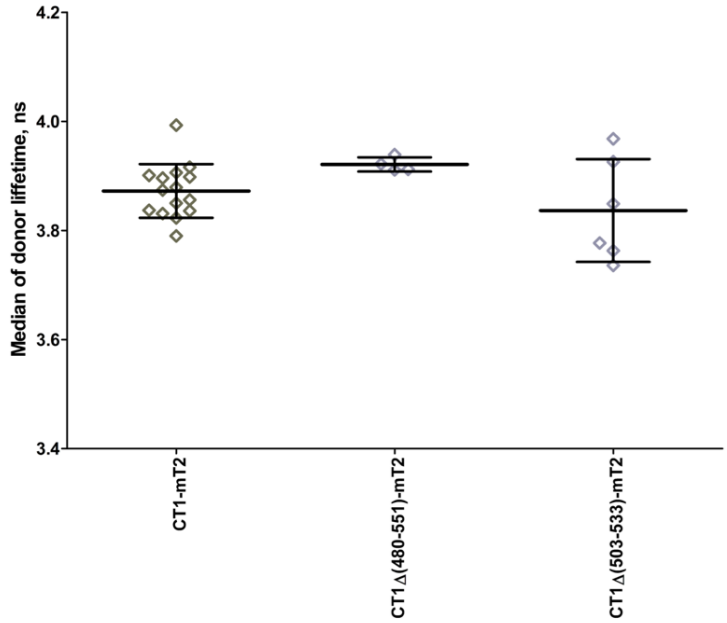

4.2
4.0
3.8
3.6
3.4
Median of donor liffetime, ns
CT1-mT2
CT1Δ(480-551)-mT2
CT1Δ(503-533)-mT2

Fig S2

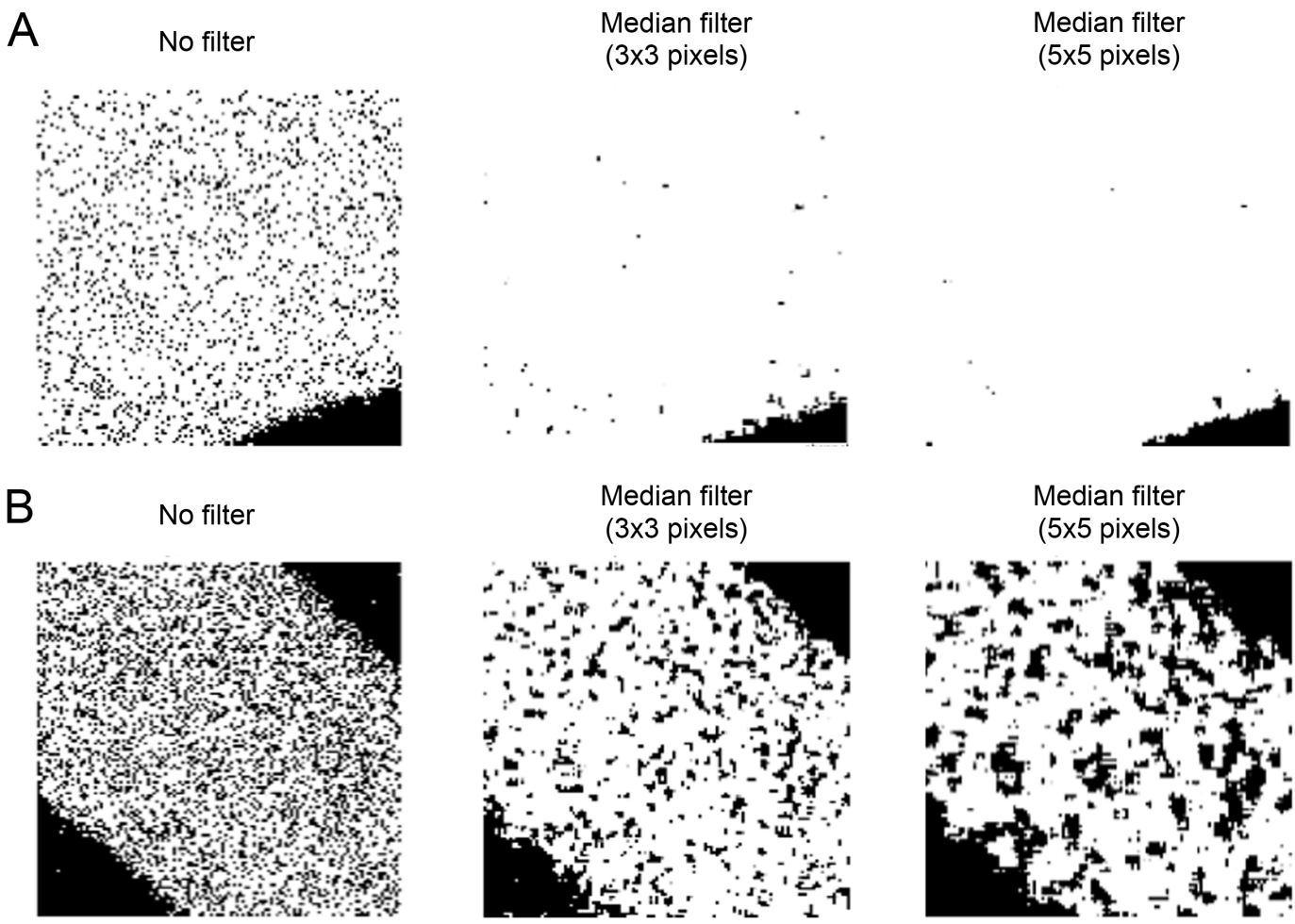

A
No filter
Median filter
(3x3 pixels)
Median filter
(5x5 pixels)
B
No filter
Median filter
(3x3 pixels)
Median filter
(5x5 pixels)

Fig S3

A

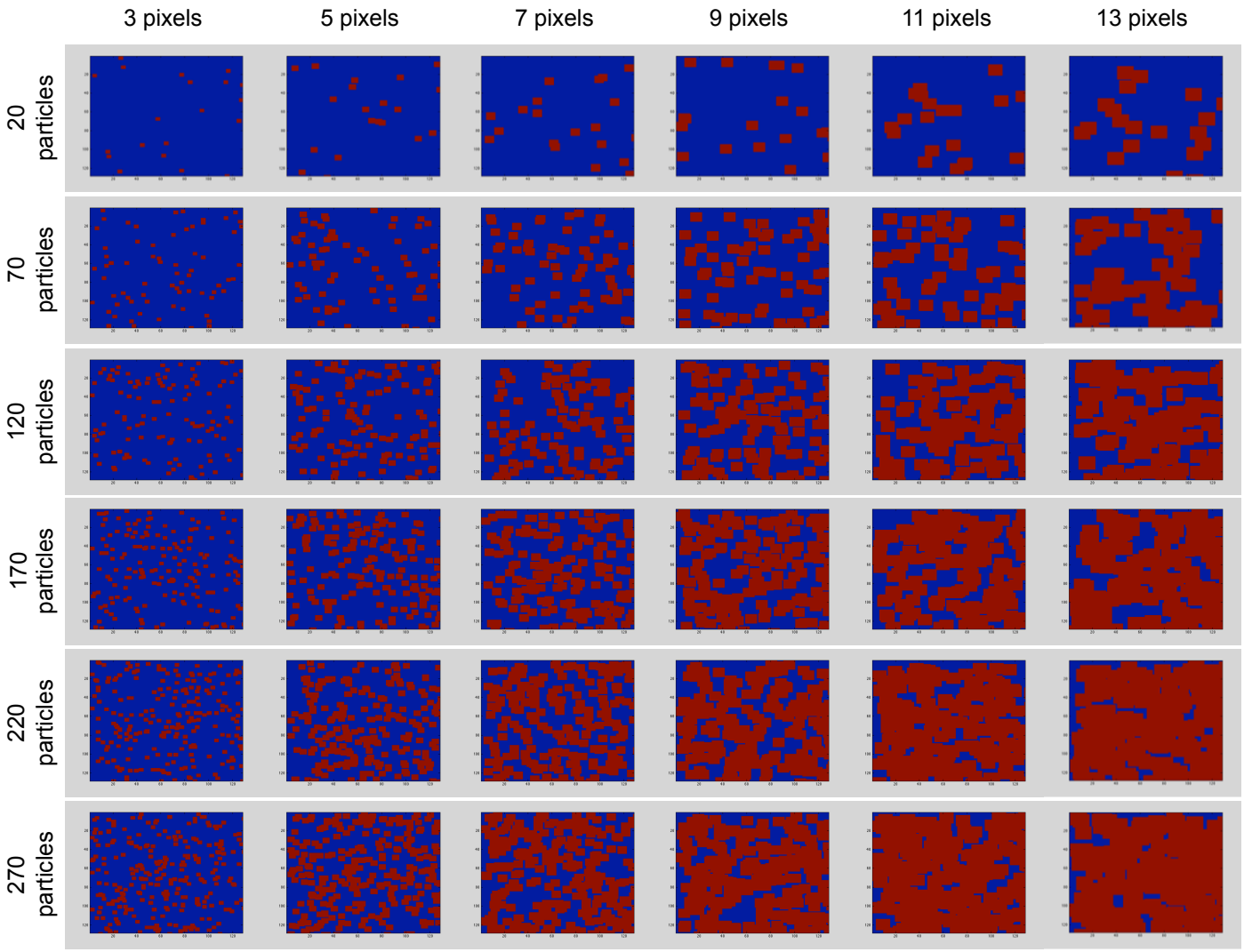
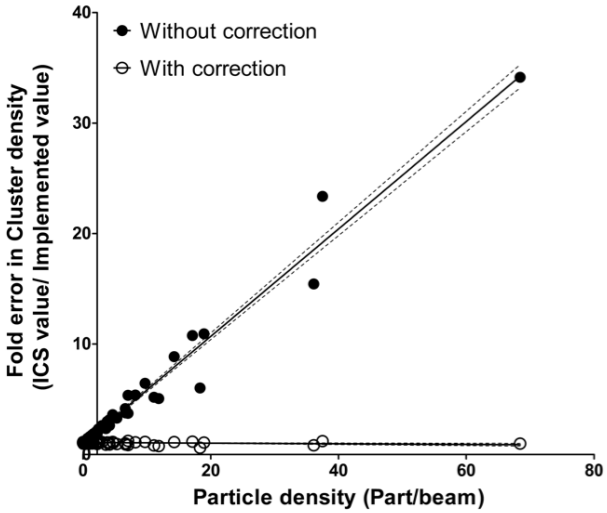

3 pixels
5 pixels
7 pixels
9 pixels
11 pixels
13 pixels
20 particles
70 particles
120 particles
170 particles
220 particles
270 particles

Fig S4

A


Without correction
With correction
Fold error in Cluster density
(ICS value/ Implemented value)
Particle density (Part/beam)
0
10
20
30
40
0
20
40
60
80

B

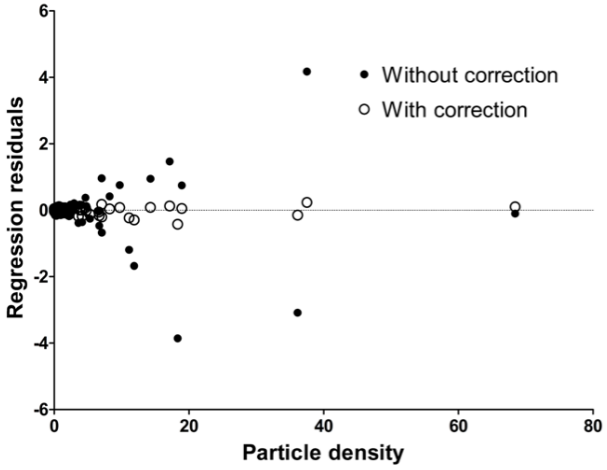

Without correction
With correction
Regression residuals
Particle density
6
4
2
0
-2
-4
-6
0
20
40
60
80

Fig S5