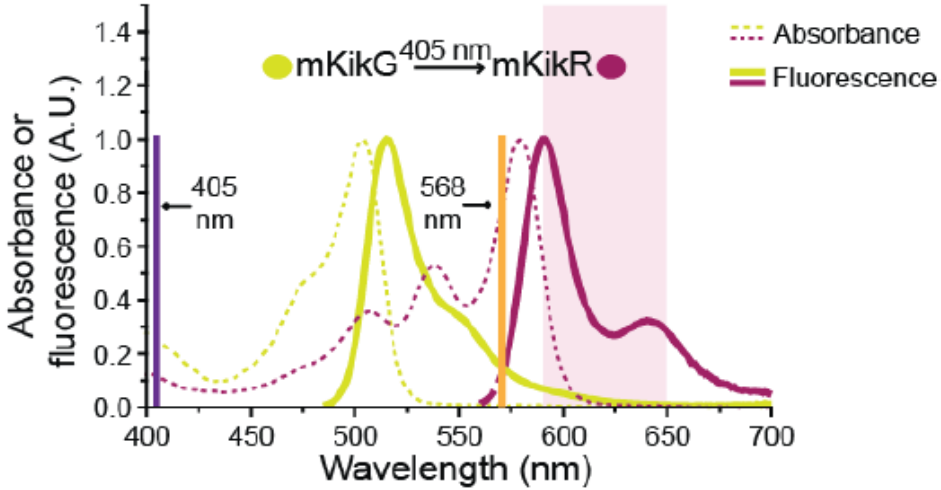
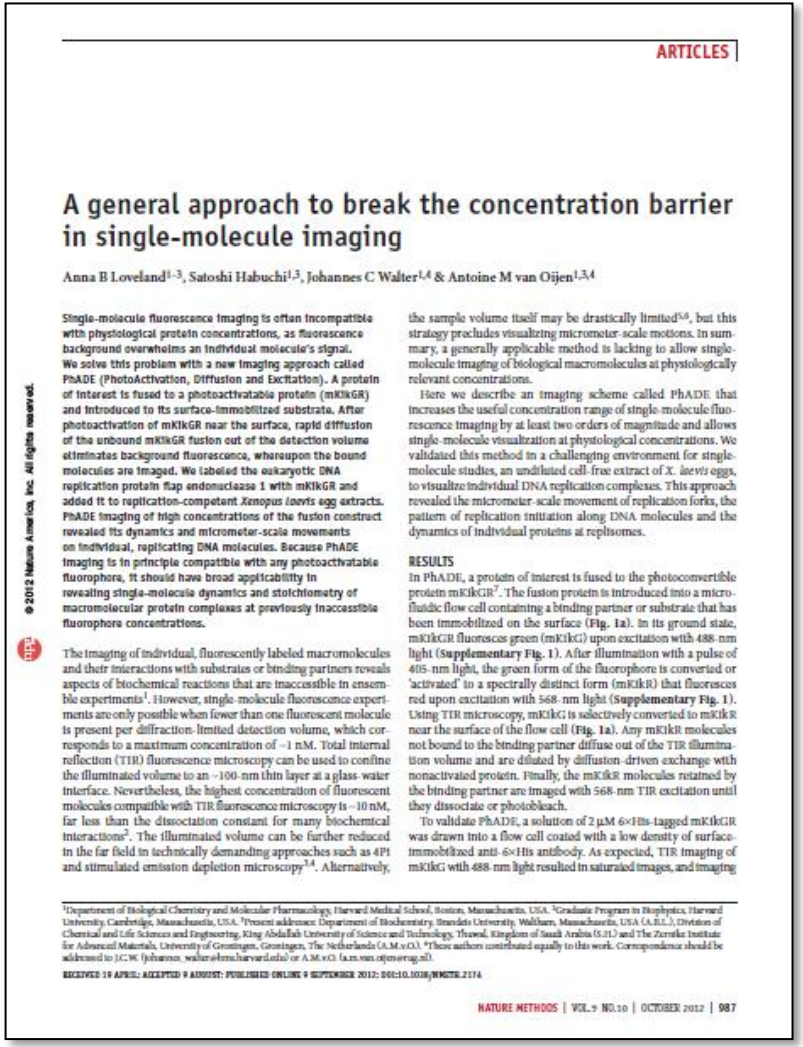


PhADE

(Photo Activation, Diffusion and Excitation)



A general approach to break the concentration barrier in single-molecule imaging

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Single-molecule fluorescence imaging is often incompatible with physiological protein concentrations, as fluorescence background overwhelms an individual molecule's signal. We solve this problem with a new imaging approach called PhADE (PhotoActivation, Diffusion and Excitation). A protein of interest is fused to a photoactivatable protein (mKikGR) and introduced to its surface-immobilized substrate. After photoactivation of mKikGR near the surface, rapid diffusion of the unbound mKikGR fusion out of the detection volume eliminates background fluorescence, whereupon the bound molecules are imaged. We labeled the eukaryotic DNA replication protein flap endonuclease 1 with mKikGR and added it to replication-competent *Xenopus laevis* egg extracts. PhADE imaging of high concentrations of the fusion construct revealed its dynamics and micrometer-scale movements on individual, replicating DNA molecules. Because PhADE imaging is in principle compatible with any photoactivatable fluorophore, it should have broad applicability in revealing single-molecule dynamics and stoichiometry of macromolecular protein complexes at previously inaccessible fluorophore concentrations.

The imaging of individual, fluorescently labeled macromolecules and their interactions with substrates or binding partners reveals aspects of biochemical reactions that are inaccessible in ensemble experiments[1]. However, single-molecule fluorescence experiments are only possible when fewer than one fluorescent molecule is present per diffraction-limited detection volume, which corresponds to a maximum concentration of ~1 nM. Total internal reflection (TIR) fluorescence microscopy can be used to confine the illuminated volume to an ~100-nm thin layer at a glass-water interface. Nevertheless, the highest concentration of fluorescent molecules compatible with TIR fluorescence microscopy is ~10 nM, far less than the dissociation constant for many biochemical interactions[2]. The illuminated volume can be further reduced in the far field in technically demanding approaches such as 4Pi and stimulated emission depletion microscopy[3,4]. Alternatively, the sample volume itself may be drastically limited[5,6], but this strategy precludes visualizing micrometer-scale motions. In summary, a generally applicable method is lacking to allow single-molecule imaging of biological macromolecules at physiologically relevant concentrations.

Here we describe an imaging scheme called PhADE that increases the useful concentration range of single-molecule fluorescence imaging by at least two orders of magnitude and allows single-molecule visualization at physiological concentrations. We validated this method in a challenging environment for single-molecule studies, an undiluted cell-free extract of *X. laevis* eggs, to visualize individual DNA replication complexes. This approach revealed the micrometer-scale movement of replication forks, the pattern of replication initiation along DNA molecules and the dynamics of individual proteins at replisomes.

RESULTS

In PhADE, a protein of interest is fused to the photoconvertible protein mKikGR[7]. The fusion protein is introduced into a microfluidic flow cell containing a binding partner or substrate that has been immobilized on the surface (**Fig. 1a**). In its ground state, mKikGR fluoresces green (mKikG) upon excitation with 488-nm light (**Supplementary Fig. 1**). After illumination with a pulse of 405-nm light, the green form of the fluorophore is converted or 'activated' to a spectrally distinct form (mKikR) that fluoresces red upon excitation with 568-nm light (**Supplementary Fig. 1**). Using TIR microscopy, mKikG is selectively converted to mKikR near the surface of the flow cell (**Fig. 1a**). Any mKikR molecules not bound to the binding partner diffuse out of the TIR illumination volume and are diluted by diffusion-driven exchange with nonactivated protein. Finally, the mKikR molecules retained by the binding partner are imaged with 568-nm TIR excitation until they dissociate or photobleach.

To validate PhADE, a solution of 2 μM 6×His-tagged mKikGR was drawn into a flow cell coated with a low density of surface-immobilized anti-6×His antibody. As expected, TIR imaging of mKikG with 488-nm light resulted in saturated images, and imaging

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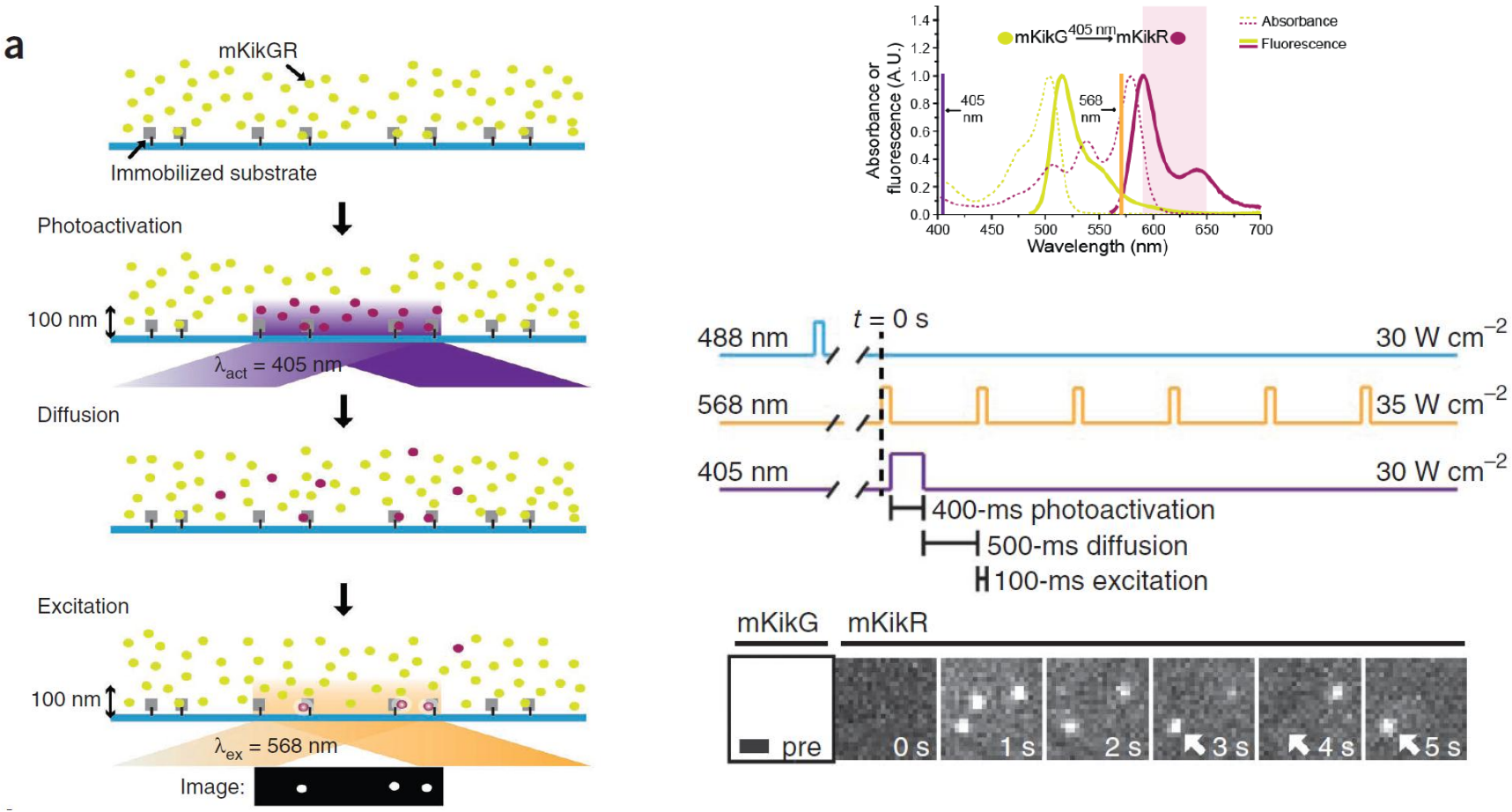
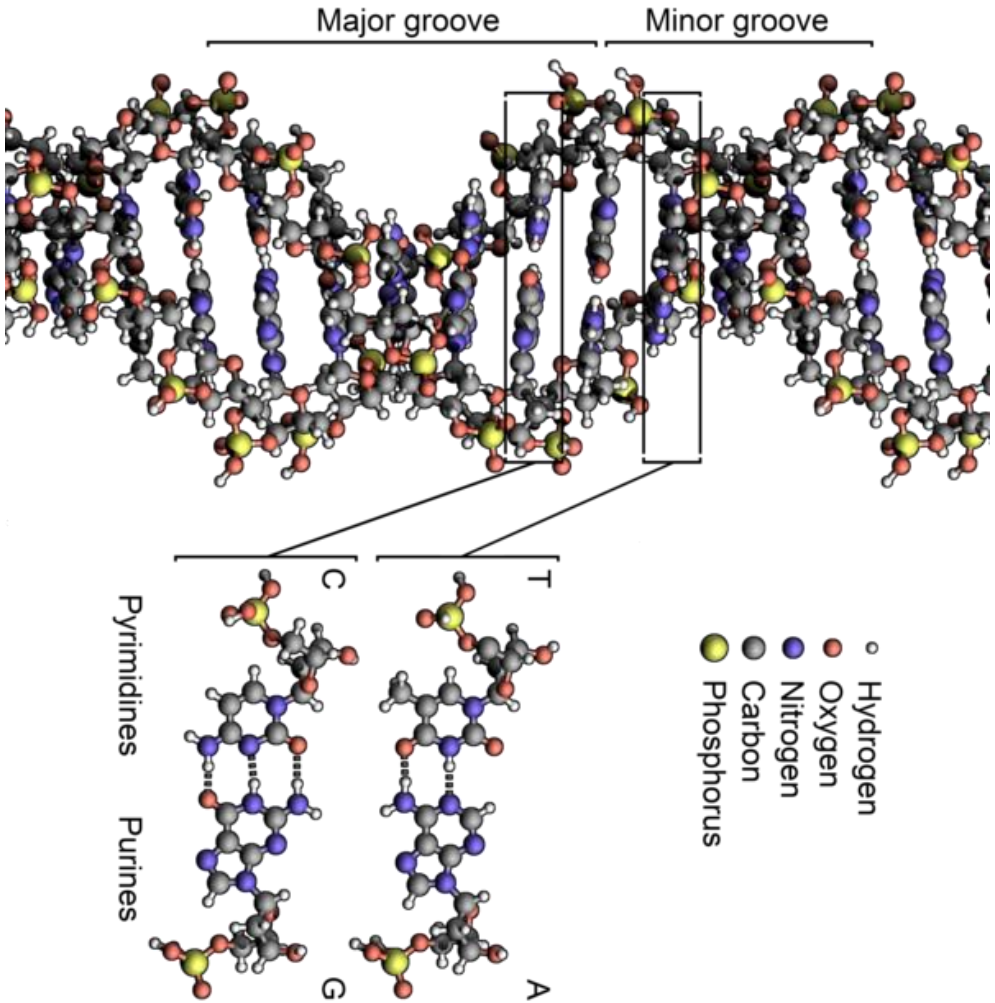
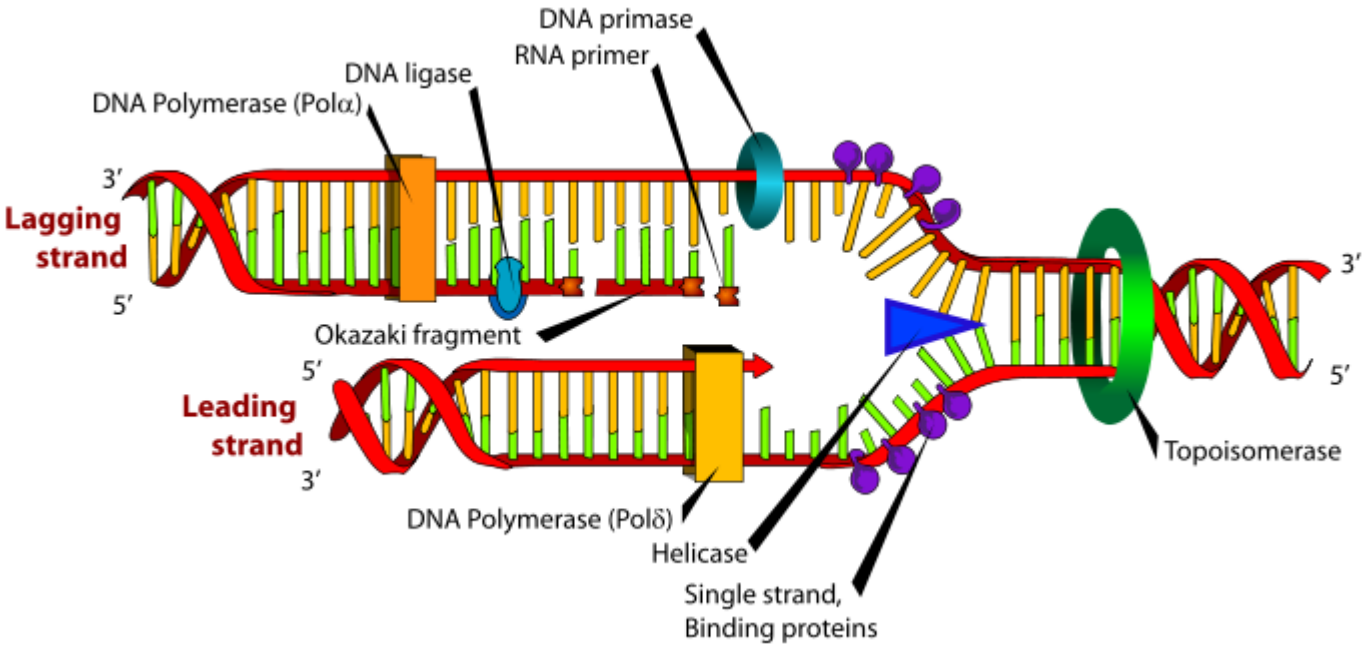

a
mKikGR
Immobilized substrate
Photoactivation
100 nm
λact = 405 nm
Diffusion
Excitation
100 nm
λex = 568 nm
Image:
mKikG 405 nm mKikR
Absorbance
Fluorescence
Absorbance or fluorescence (A.U.)
405 nm
568 nm
Wavelength (nm)
488 nm
t = 0 s
30 W cm−2
568 nm
35 W cm−2
405 nm
30 W cm−2
400-ms photoactivation
500-ms diffusion
100-ms excitation
mKikG
mKikR
pre
0 s
1 s
2 s
3 s
4 s
5 s

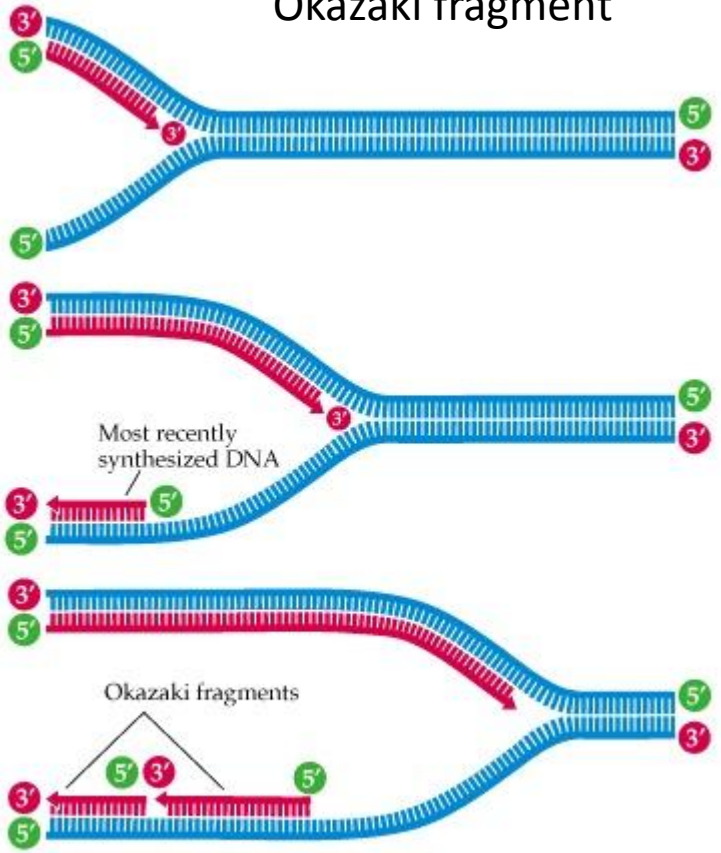


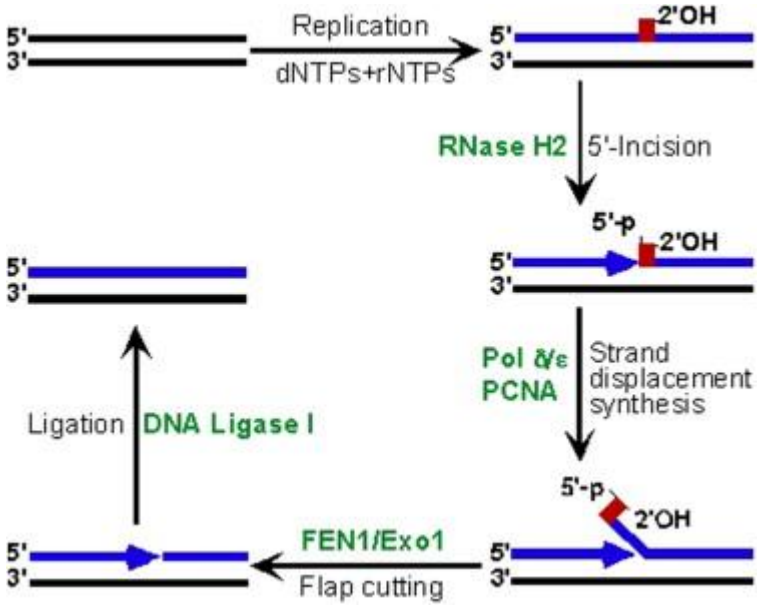
http://en.wikipedia.org/wiki/File:DNA_Structure%2BKey%2BLabelled.pn_NoBB.png


DNA primase
RNA primer
DNA ligase
DNA Polymerase (Polα)
3′
Lagging strand
5′
Okazaki fragment
5′
Leading strand
3′
DNA Polymerase (Polδ)
Helicase
Single strand,
Binding proteins
Topoisomerase
3′
5′

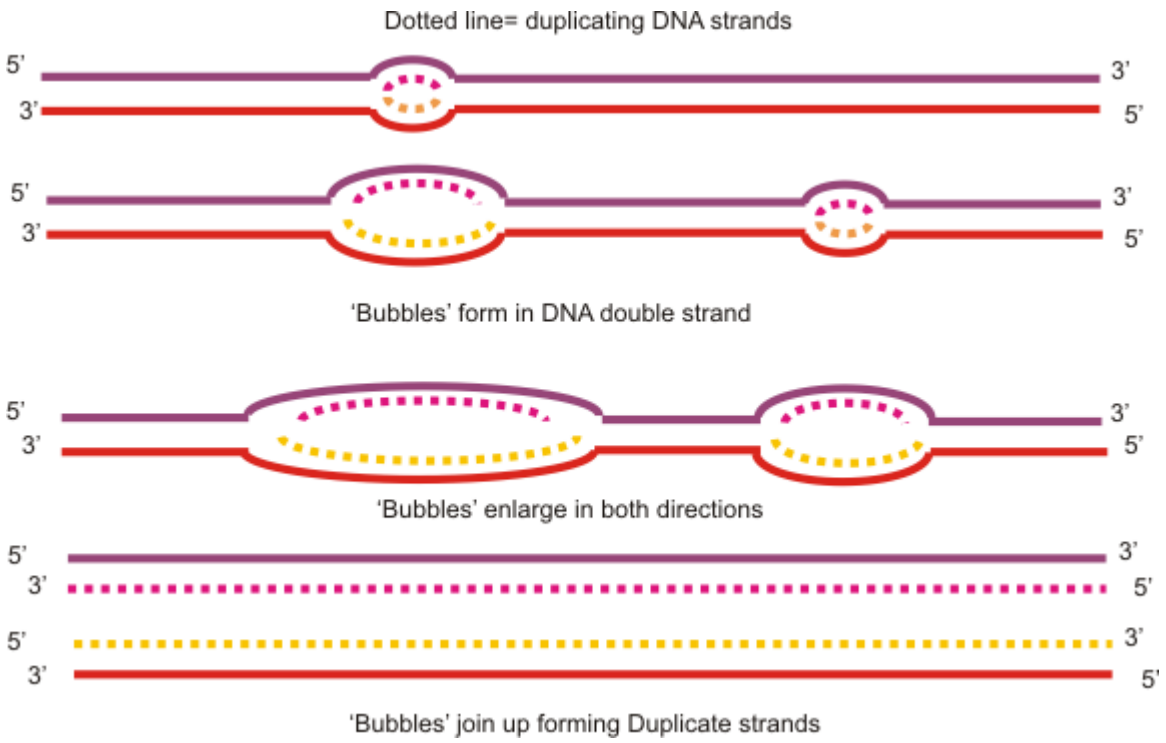
http://www.youtube.com/watch?v=teV62zrm2P0

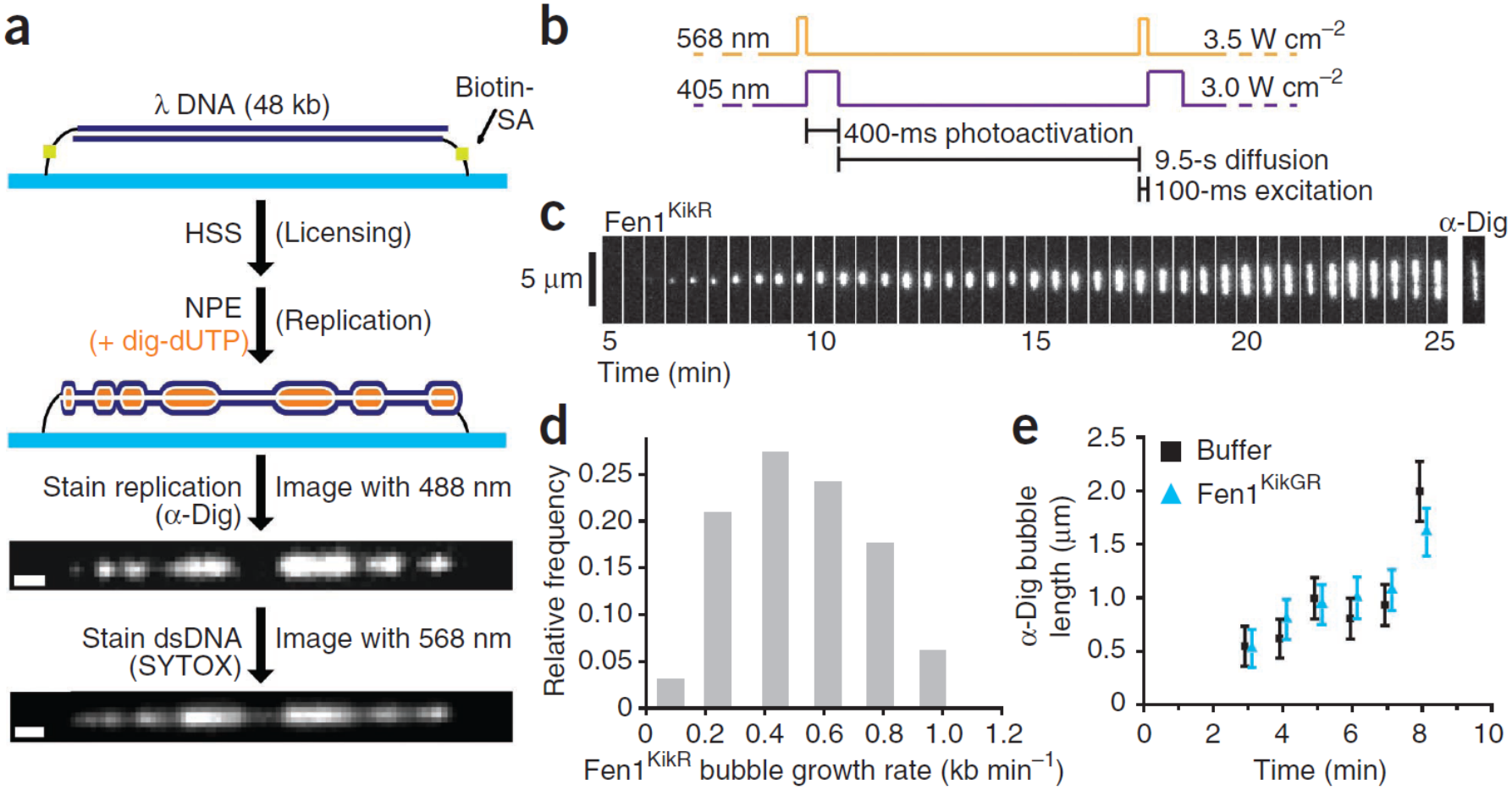
Okazaki fragment

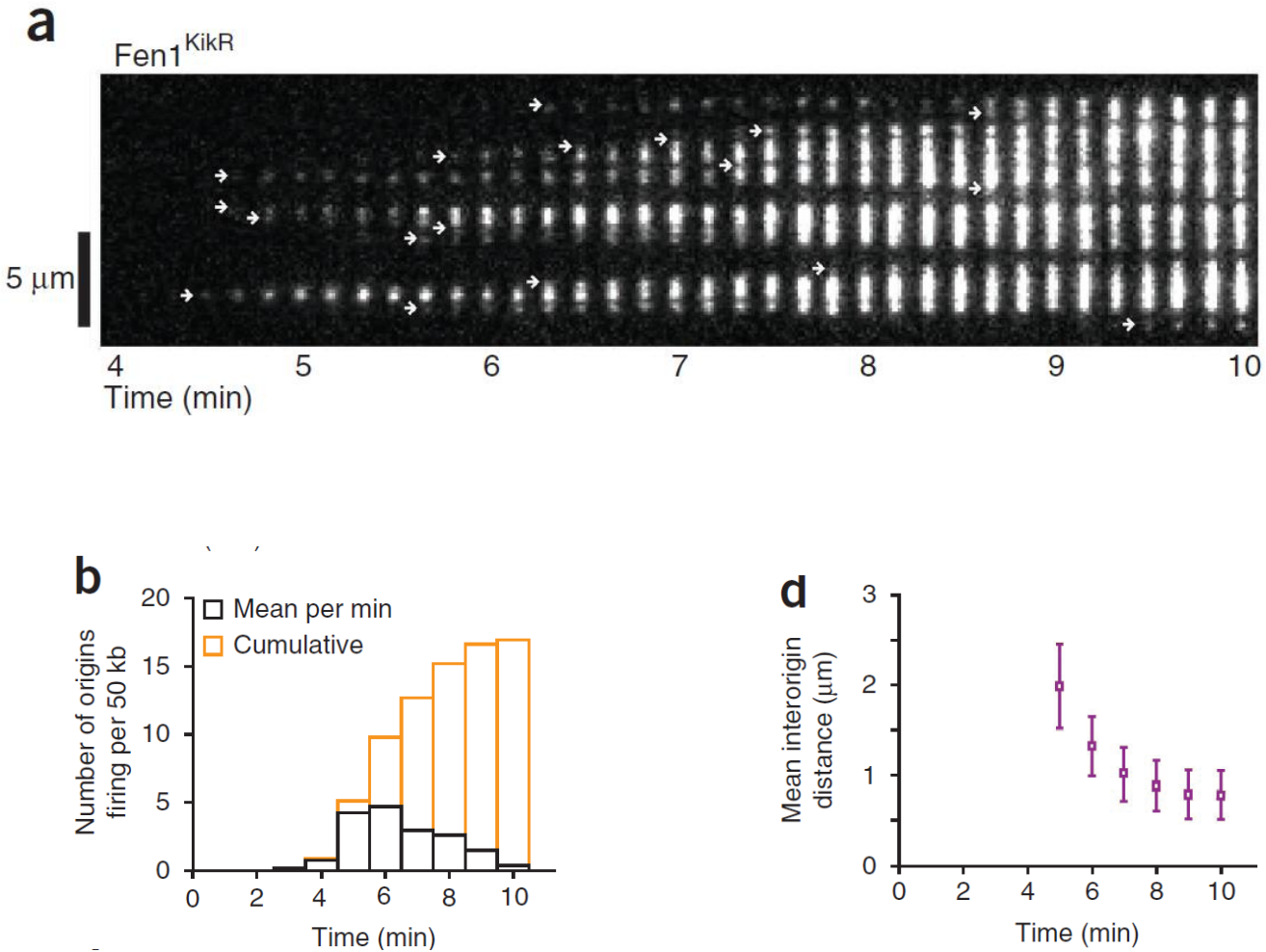


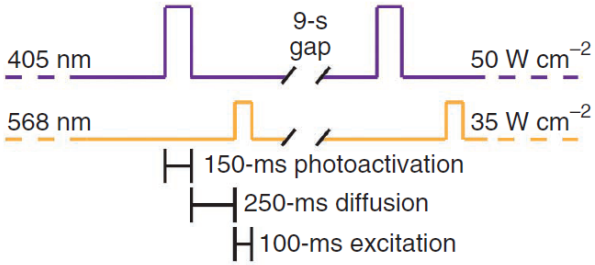


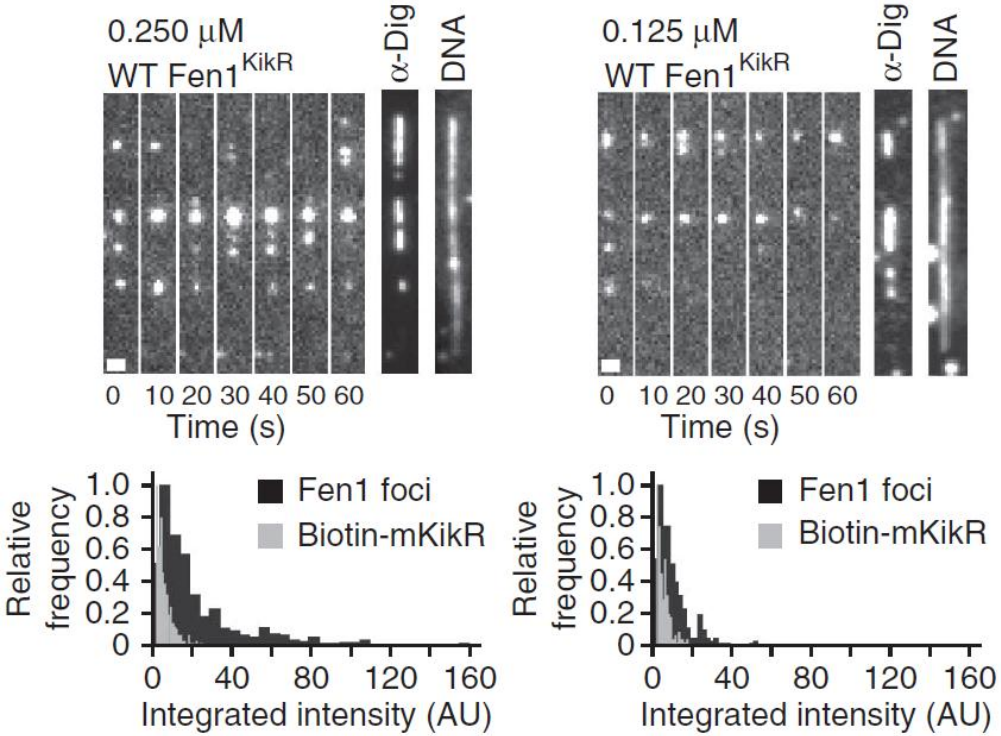
Replication bubble

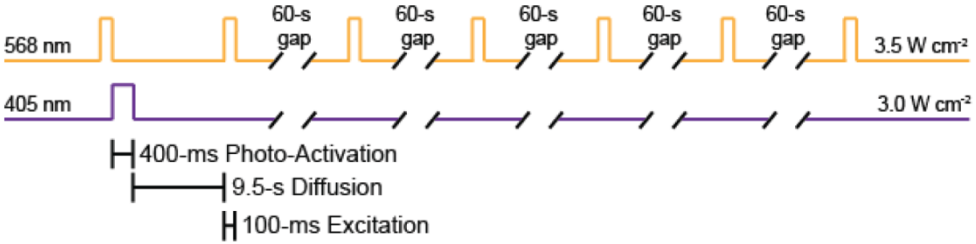


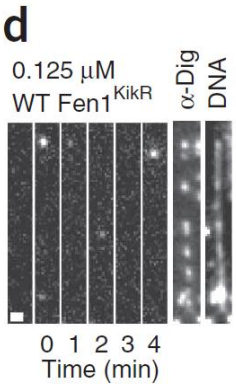

a
λ DNA (48 kb)
Biotin-SA
HSS
(Licensing)
NPE
(+ dig-dUTP)
(Replication)
Stain replication
(α-Dig)
Image with 488 nm
Stain dsDNA
(SYTOX)
Image with 568 nm
b
568 nm
3.5 W cm−2
405 nm
3.0 W cm−2
400-ms photoactivation
9.5-s diffusion
100-ms excitation
c
Fen1KikR
α-Dig
5 μm
5
10
15
20
25
Time (min)
d
Relative frequency
0.25
0.20
0.15
0.10
0.05
0
0
0.2
0.4
0.6
0.8
1.0
1.2
Fen1KikR bubble growth rate (kb min−1)
e
Buffer
Fen1KikGR
α-Dig bubble length (μm)
2.5
2.0
1.5
1.0
0.5
0
0
2
4
6
8
10
Time (min)

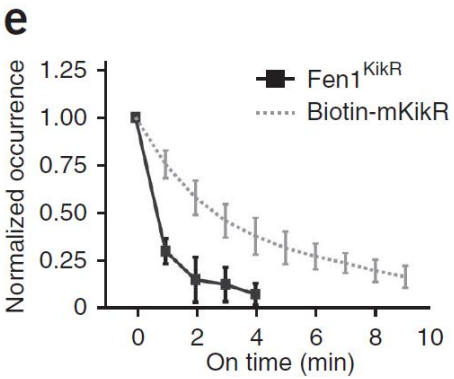

a
Fen1KikR
5 µm
4
5
6
7
8
9
10
Time (min)
b
Mean per min
Cumulative
Number of origins firing per 50 kb
20
15
10
5
0
0
2
4
6
8
10
Time (min)
d
Mean interorigin distance (µm)
3
2
1
0
0
2
4
6
8
10
Time (min)

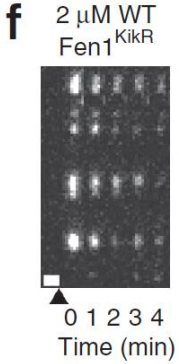

405 nm
9-s gap
50 W cm^{-2}
568 nm
35 W cm^{-2}
150-ms photoactivation
250-ms diffusion
100-ms excitation

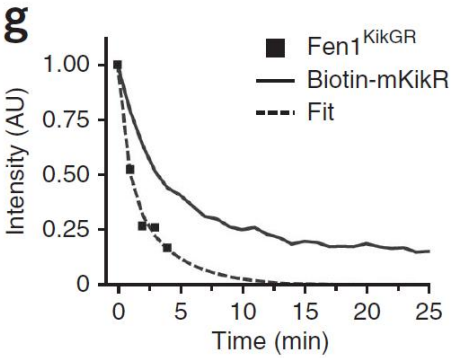

0.250 μM
WT Fen1^{KikR}
α-Dig
DNA
0 10 20 30 40 50 60
Time (s)
0.125 μM
WT Fen1^{KikR}
α-Dig
DNA
0 10 20 30 40 50 60
Time (s)
Relative frequency
1.0
0.8
0.6
0.4
0.2
0
Fen1 foci
Biotin-mKikR
0 40 80 120 160
Integrated intensity (AU)
Relative frequency
1.0
0.8
0.6
0.4
0.2
0
Fen1 foci
Biotin-mKikR
0 40 80 120 160
Integrated intensity (AU)


568 nm
60-s gap
3.5 W cm-2
405 nm
3.0 W cm-2
400-ms Photo-Activation
9.5-s Diffusion
100-ms Excitation


d
0.125 μM
WT Fen1KikR
α-Dig
DNA
0 1 2 3 4
Time (min)


e
Fen1KikR
Biotin-mKikR
Normalized occurrence
1.25
1.00
0.75
0.50
0.25
0
0 2 4 6 8 10
On time (min)


f
2 μM WT
Fen1KikR
0 1 2 3 4
Time (min)


g
Fen1KikGR
Biotin-mKikR
Fit
Intensity (AU)
1.00
0.75
0.50
0.25
0
0 5 10 15 20 25
Time (min)