LETTER

doi:10.1038/nature11598

Direct imaging of RecA nucleation and growth on single molecules of SSB-coated ssDNA

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***Escherichia coli* RecA is the defining member of a ubiquitous class of DNA strand-exchange proteins that are essential for homologous recombination, a pathway that maintains genomic integrity by repairing broken DNA[1]. To function, filaments of RecA must nucleate and grow on single-stranded DNA (ssDNA) in direct competition with ssDNA-binding protein (SSB), which rapidly binds and continuously sequesters ssDNA, kinetically blocking RecA assembly[2,3]. This dynamic self-assembly on a DNA lattice, in competition with another protein, is unique for the RecA family compared to other filament-forming proteins such as actin and tubulin. The complexity of this process has hindered our understanding of RecA filament assembly because ensemble measurements cannot reliably distinguish between the nucleation and growth phases, despite extensive and diverse attempts[2–5]. Previous single-molecule assays have measured the nucleation and growth of RecA—and its eukaryotic homologue RAD51—on naked double-stranded DNA and ssDNA[6–12]; however, the template for RecA self-assembly *in vivo* is SSB-coated ssDNA[1]. Using single-molecule microscopy, here we directly visualize RecA filament assembly on single molecules of SSB-coated ssDNA, simultaneously measuring nucleation and growth. We establish that a dimer of RecA is required for nucleation, followed by growth of the filament through monomer addition, consistent with the finding that nucleation, but not growth, is modulated by nucleotide and magnesium ion cofactors. Filament growth is bidirectional, albeit faster in the 5′→3′ direction. Both nucleation and growth are repressed at physiological conditions, highlighting the essential role of recombination mediators in potentiating assembly *in vivo*. We define a two-step kinetic mechanism in which RecA nucleates on transiently exposed ssDNA during SSB sliding and/or partial dissociation (DNA unwrapping) and then the RecA filament grows. We further demonstrate that the recombination mediator protein pair, RecOR (RecO and RecR), accelerates both RecA nucleation and filament growth, and that the introduction of RecF further stimulates RecA nucleation.**

To image the assembly of RecA filaments on SSB-coated ssDNA, we first developed a procedure to generate and visualize single molecules of ssDNA. Bacteriophage λ double-stranded DNA (dsDNA) 48.5 kilobase pairs (kb) long was biotinylated at the 3′ ends, alkali denatured, neutralized with buffer and saturated with a fluorescently modified SSB (SSB^{AF488}). This complex was then attached to the surface of a streptavidin-coated glass coverslip within a microfluidic chamber, extended by buffer flow, and visualized using total internal reflection fluorescence (TIRF) microscopy (Fig. 1a, b, top panels). Because the binding affinity of SSB^{AF488} is attenuated[13,14], we next replaced it with unlabelled wild-type SSB *in situ* (Fig. 1a, b, second panels, and Supplementary Video 1). The exchange of proteins in the flow cell is fast, with a half-time of approximately 2–3 s, resulting in a non-fluorescent native SSB–ssDNA complex. RecA filament assembly on the wild-type SSB–ssDNA complexes was then imaged using a fluorescent RecA protein, fluorescein-RecA ($RecA^f$), described previously[7]. Assembly was initiated by injecting $RecA^f$, free SSB, and either ATP (plus an ATP regenerating system) or the non-hydrolysable ATP analogue, ATPγS. RecA filament formation occurred slowly (Fig. 1a, b, third and subsequent panels), first appearing as a single spot (referred to as a cluster herein). Molecules were imaged intermittently over the course of 1–2 h; when the molecules were not being imaged, both the laser excitation and flow were turned off to minimize photobleaching, reduce sample volumes, and allow filament assembly to proceed on SSB-coated ssDNA in its relaxed state. With time, the nascent clusters elongated and new clusters appeared; these mixed nucleoprotein complexes comprised relatively stiff, rod-like RecA filament clusters interspersed between compacted and flexible SSB-coated ssDNA[15]. The composition of these intermediates was confirmed using atomic force microscopy (Supplementary Fig. 1). At the flow rate used, the SSB–ssDNA complexes are compacted to approximately 15% of the corresponding length

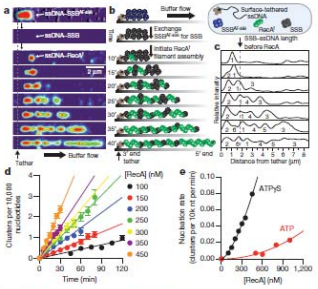

Figure 1 | Direct visualization of RecA filament assembly on single molecules of SSB-coated ssDNA shows that RecA nucleates as a dimer. **a**, $RecA^f$ filament assembly with ATPγS on a single molecule of SSB-coated ssDNA tethered within a microfluidic flow chamber was visualized using TIRF microscopy; montage is rendered into a topographic fluorescent intensity map **b**, **c**, Schematic (**b**) and fluorescent intensity profile (**c**) from **a**. **d**, The number of RecA clusters increases linearly with time (shown in minutes); slope is the nucleation rate ($n = 18$–93 clusters for each concentration; ± s.d.). **e**, Nucleation rate increases with RecA concentration ([RecA]) according to the equation $J = k[\mathrm{RecA}]^n$, in which n is 2.2 ± 0.6 for ATPγS and 1.5 ± 0.1 for ATP (the error from the linear fits in **d** is smaller than the symbols). nt, nucleotides.

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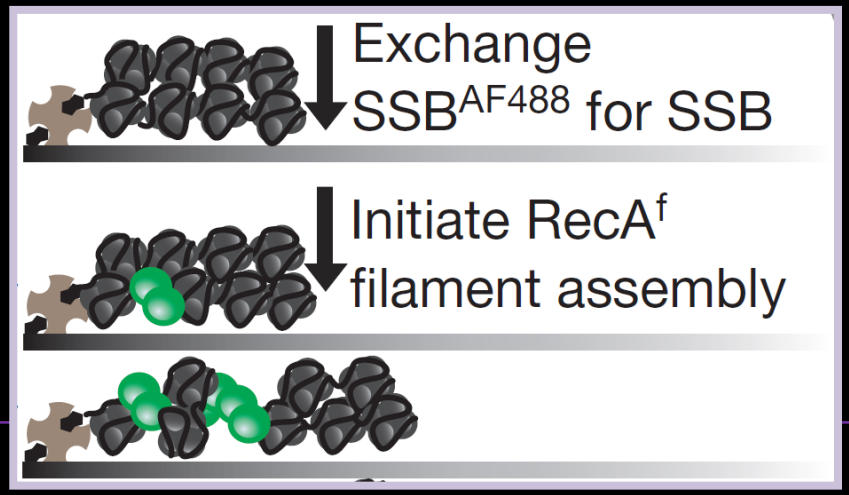
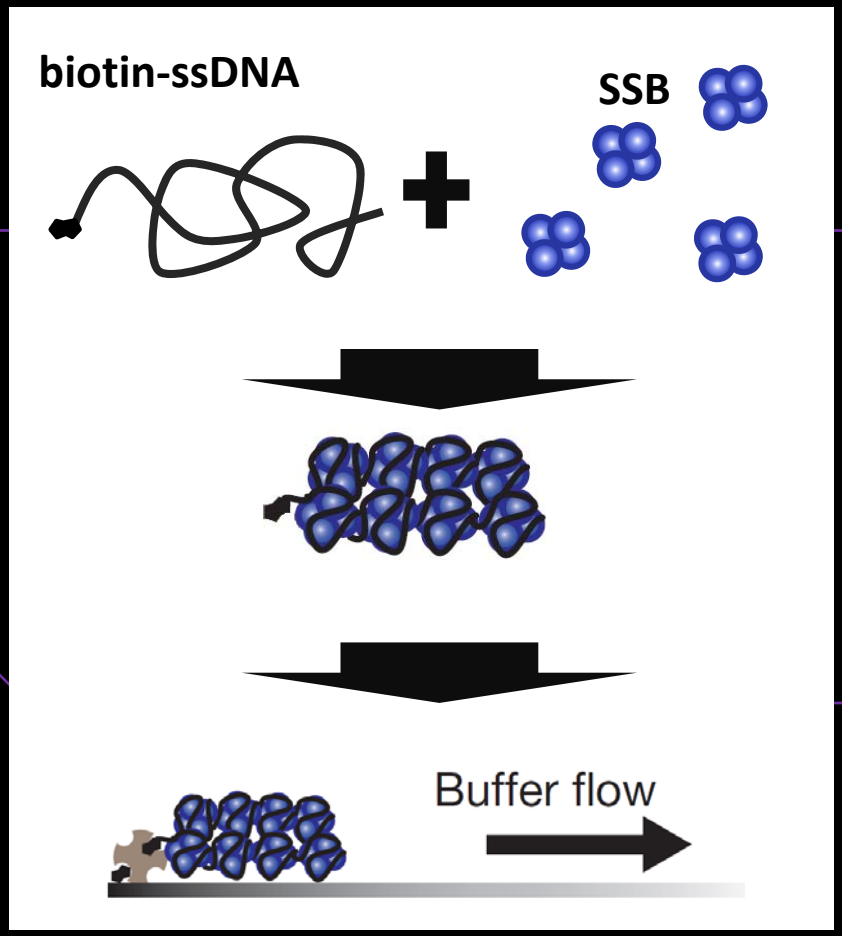
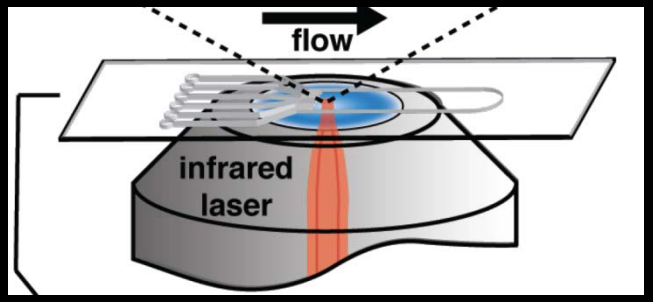
RecA vs. SSB

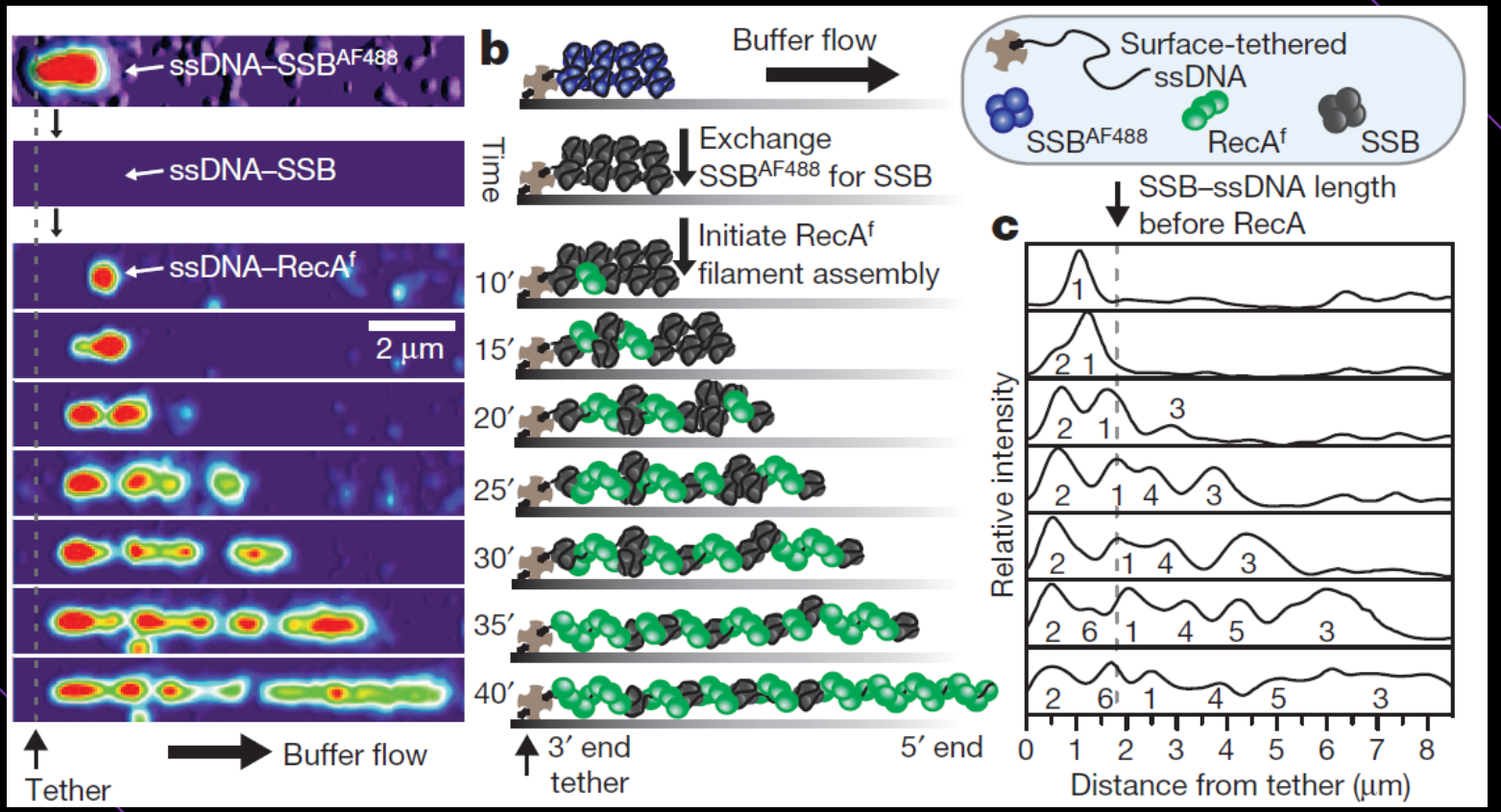


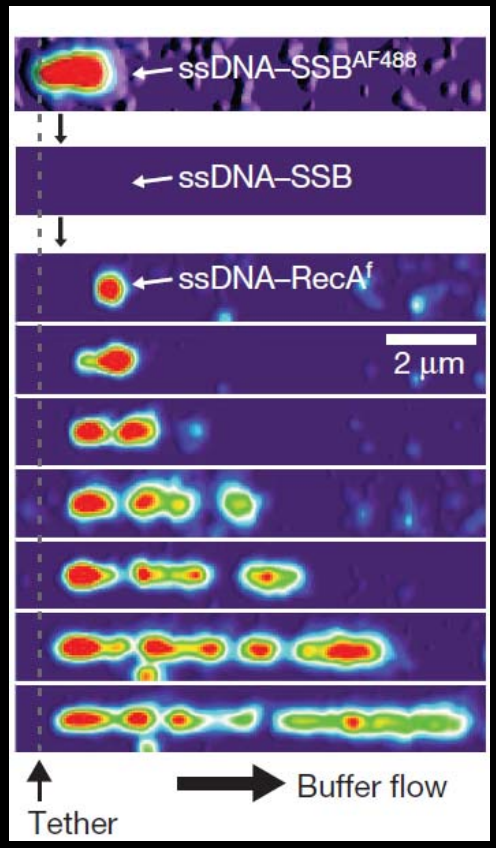
sample preparation

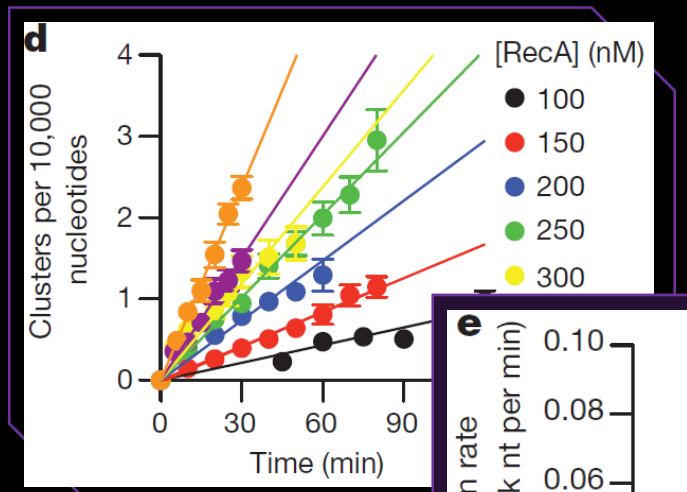


observation




ssDNA–SSB^AF488
ssDNA–SSB
ssDNA–RecA^f
2 μm
Tether
Buffer flow
b
Buffer flow
Time
Exchange SSB^AF488 for SSB
Initiate RecA^f filament assembly
10′
15′
20′
25′
30′
35′
40′
3′ end tether
5′ end
Surface-tethered ssDNA
SSB^AF488
RecA^f
SSB
c
SSB–ssDNA length before RecA
Relative intensity
Distance from tether (μm)
0 1 2 3 4 5 6 7 8

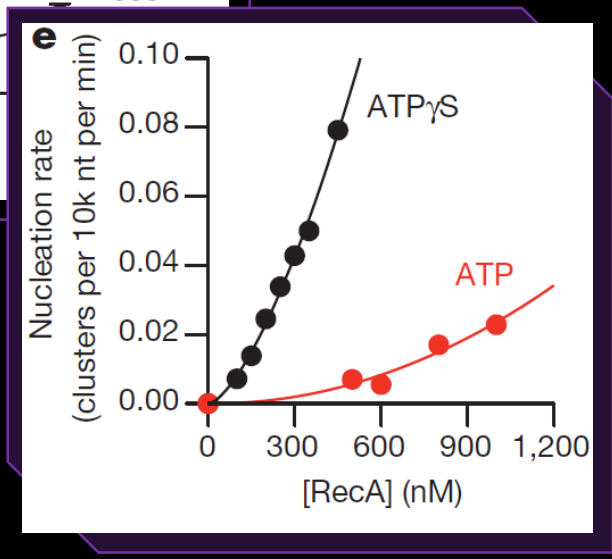


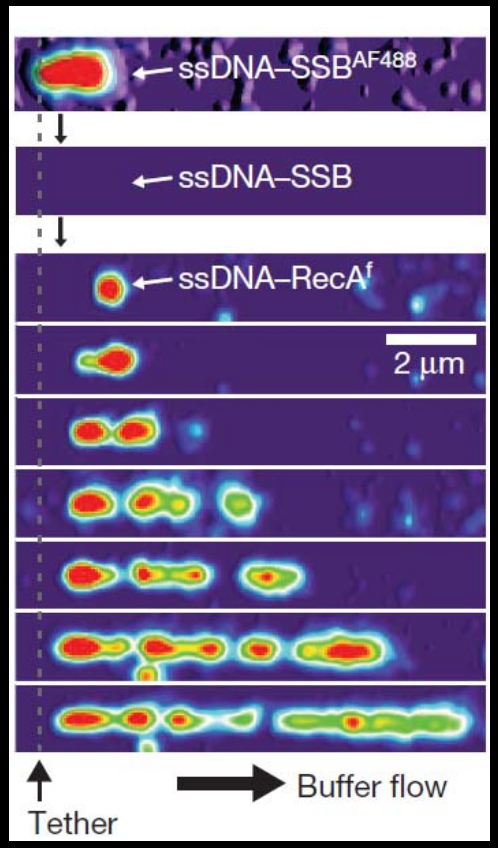


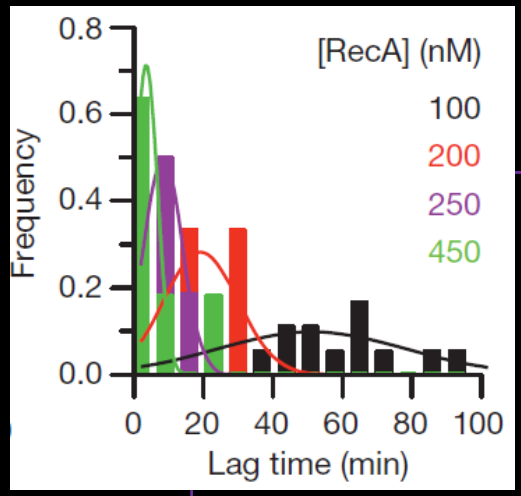
$$J = k[RecA]^n$$

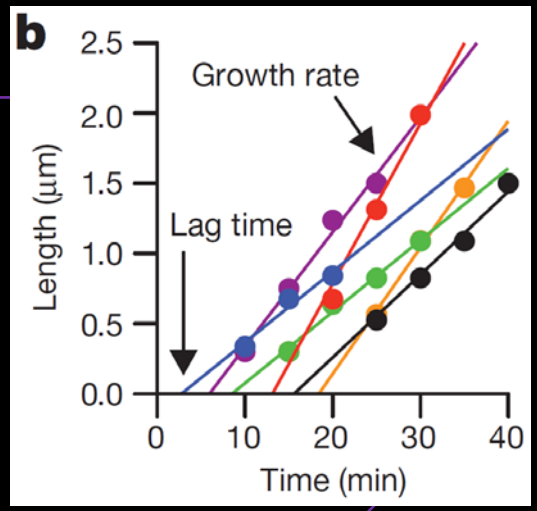
n=2.2 ± 0.6 (ATPγS)

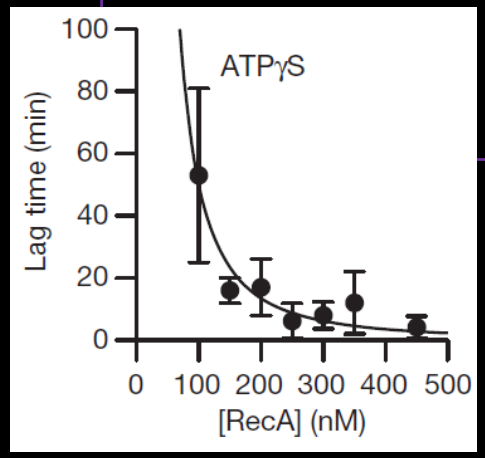
n=1.5 ± 0.1 (ATP)

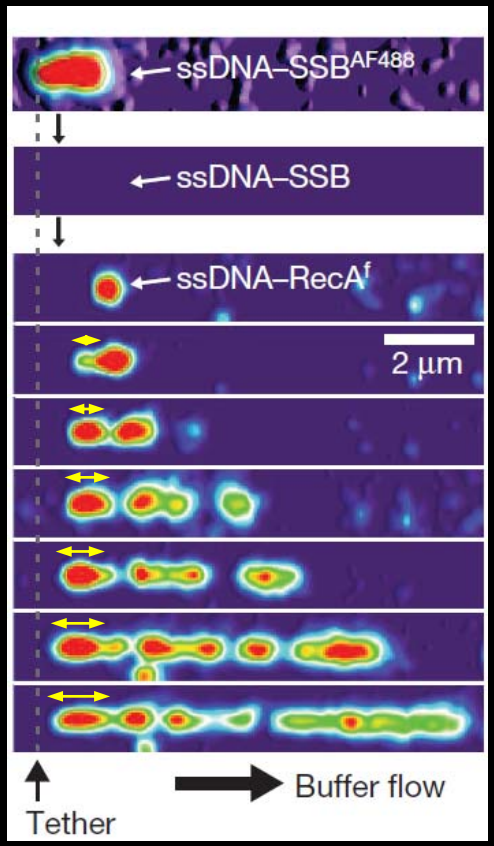


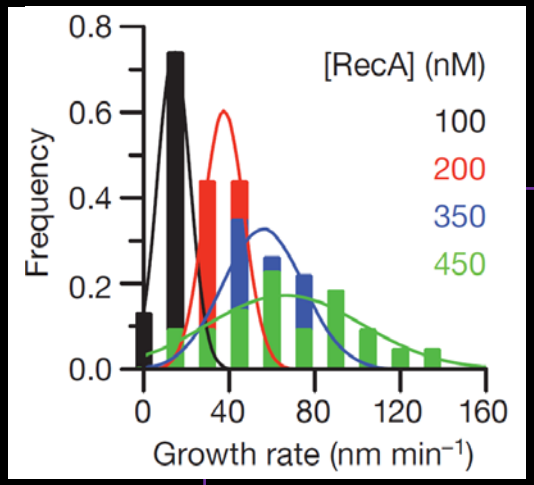

ssDNA–SSB^{AF488}
ssDNA–SSB
ssDNA–RecA^{f}
2 µm
Tether
Buffer flow

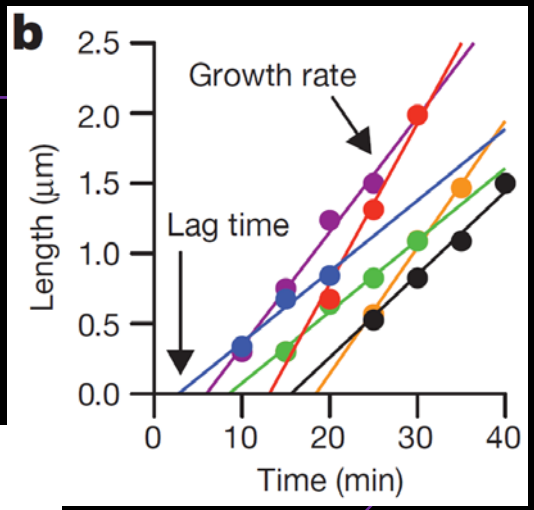

[RecA] (nM)
100
200
250
450
Frequency
0.0
0.2
0.4
0.6
0.8
0
20
40
60
80
100
Lag time (min)

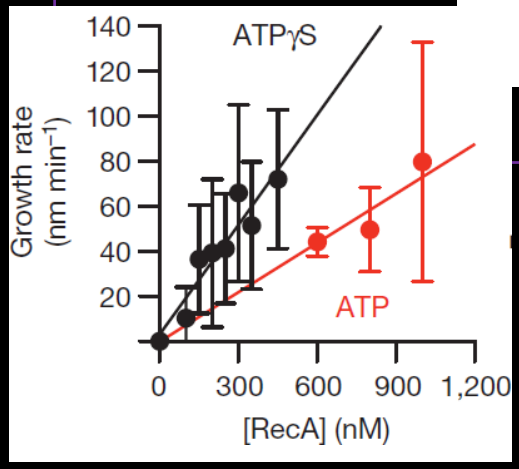

b
Growth rate
Lag time
Length (µm)
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
Time (min)

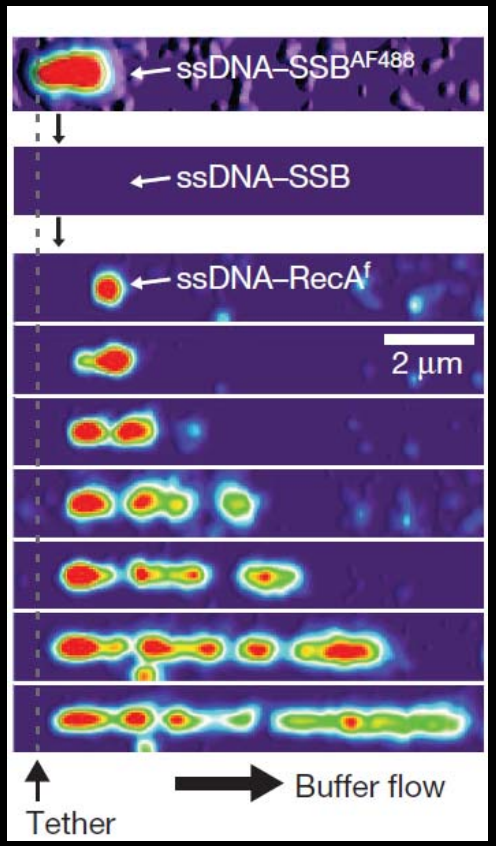

ATPγS
Lag time (min)
0
20
40
60
80
100
0
100
200
300
400
500
[RecA] (nM)

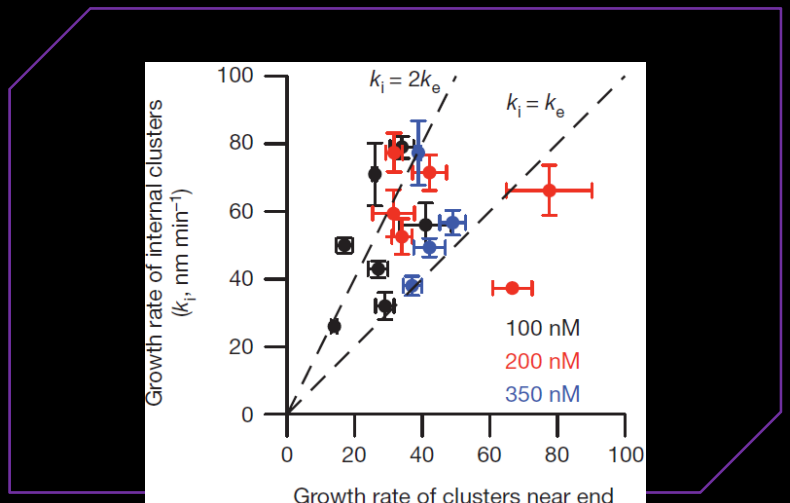

ssDNA–SSB^{AF488}
ssDNA–SSB
ssDNA–RecA^{f}
2 µm
Tether
Buffer flow

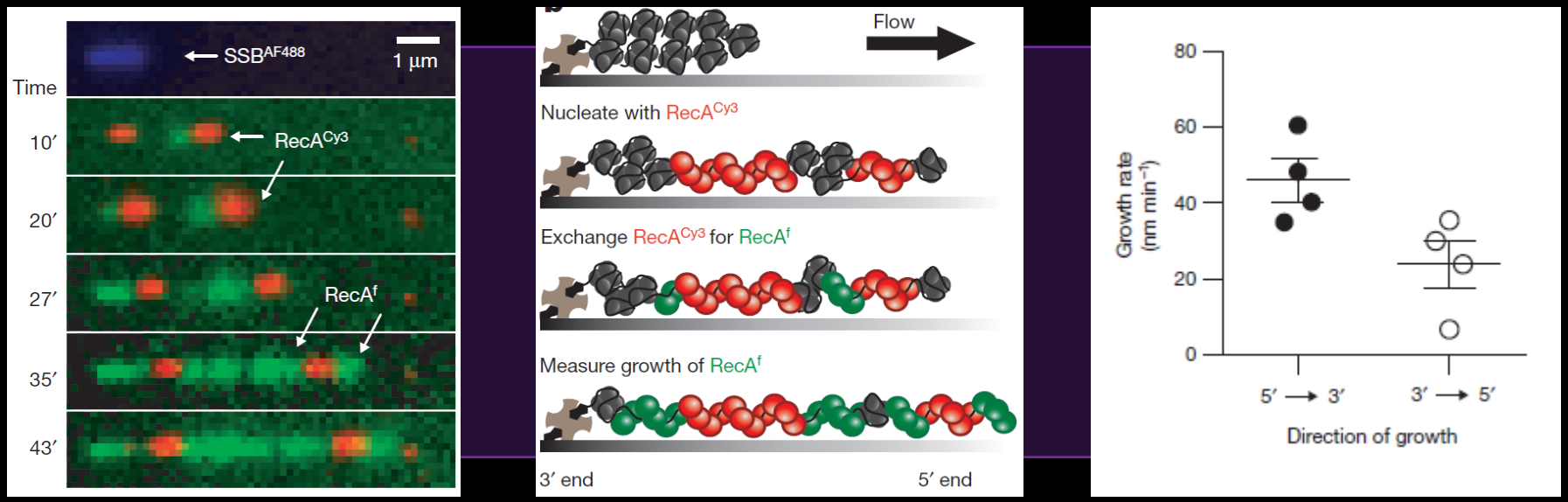

[RecA] (nM)
100
200
350
450
Frequency
0.0
0.2
0.4
0.6
0.8
0
40
80
120
160
Growth rate (nm min^{-1})

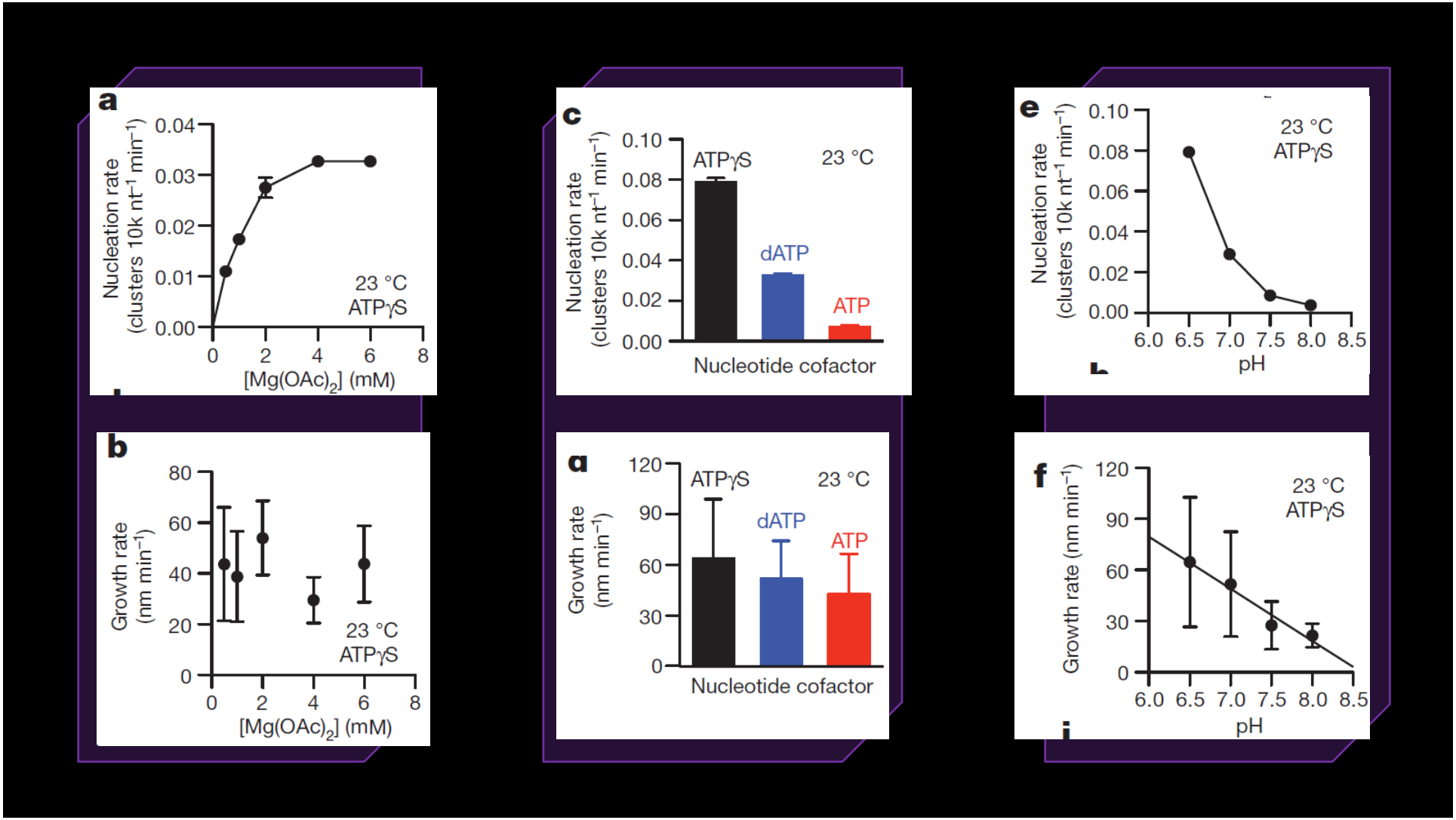

b
Growth rate
Lag time
Length (µm)
0.0
0.5
1.0
1.5
2.0
2.5
0
10
20
30
40
Time (min)

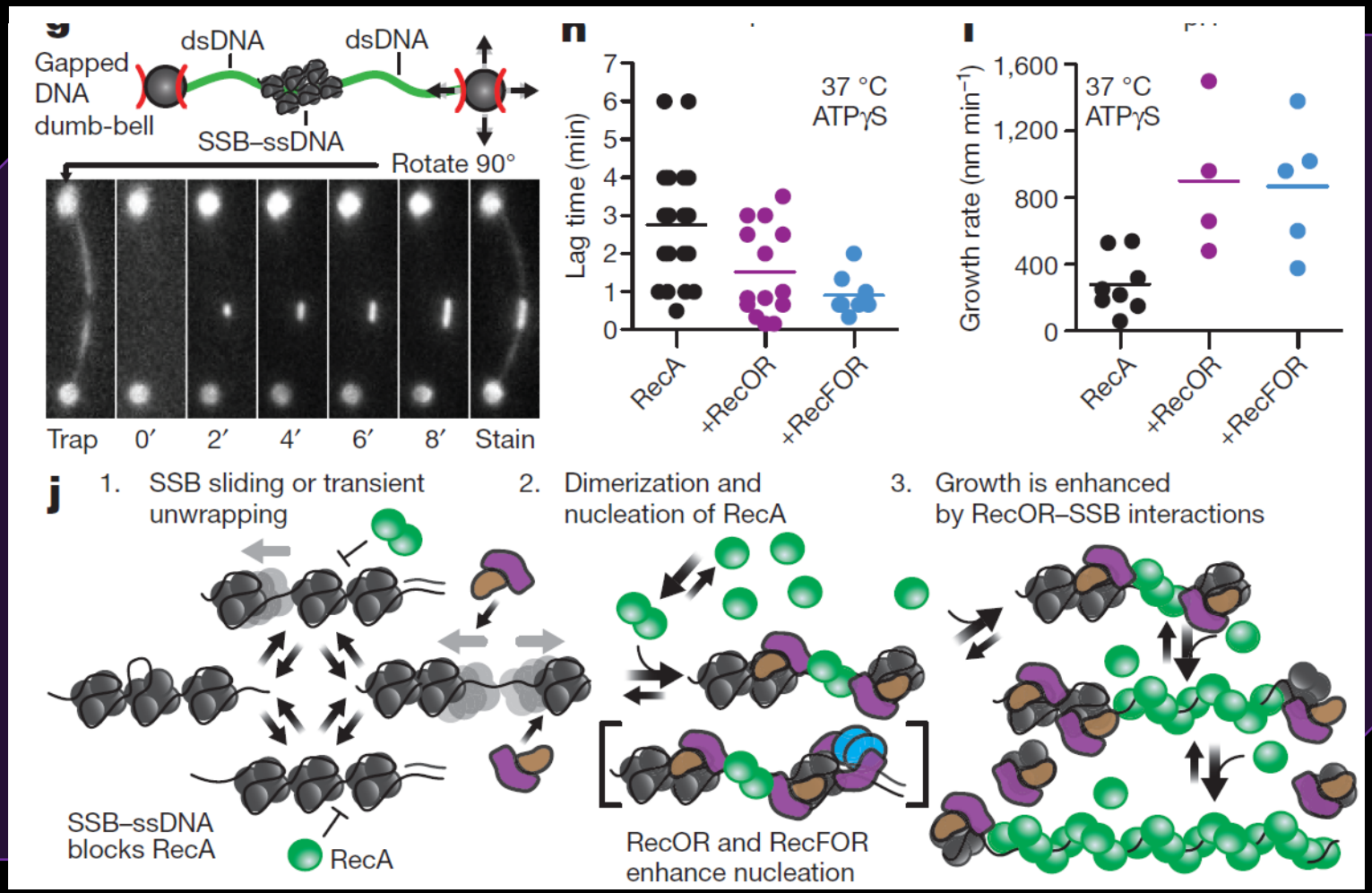

ATPγS
ATP
Growth rate (nm min^{-1})
20
40
60
80
100
120
140
0
300
600
900
1,200
[RecA] (nM)

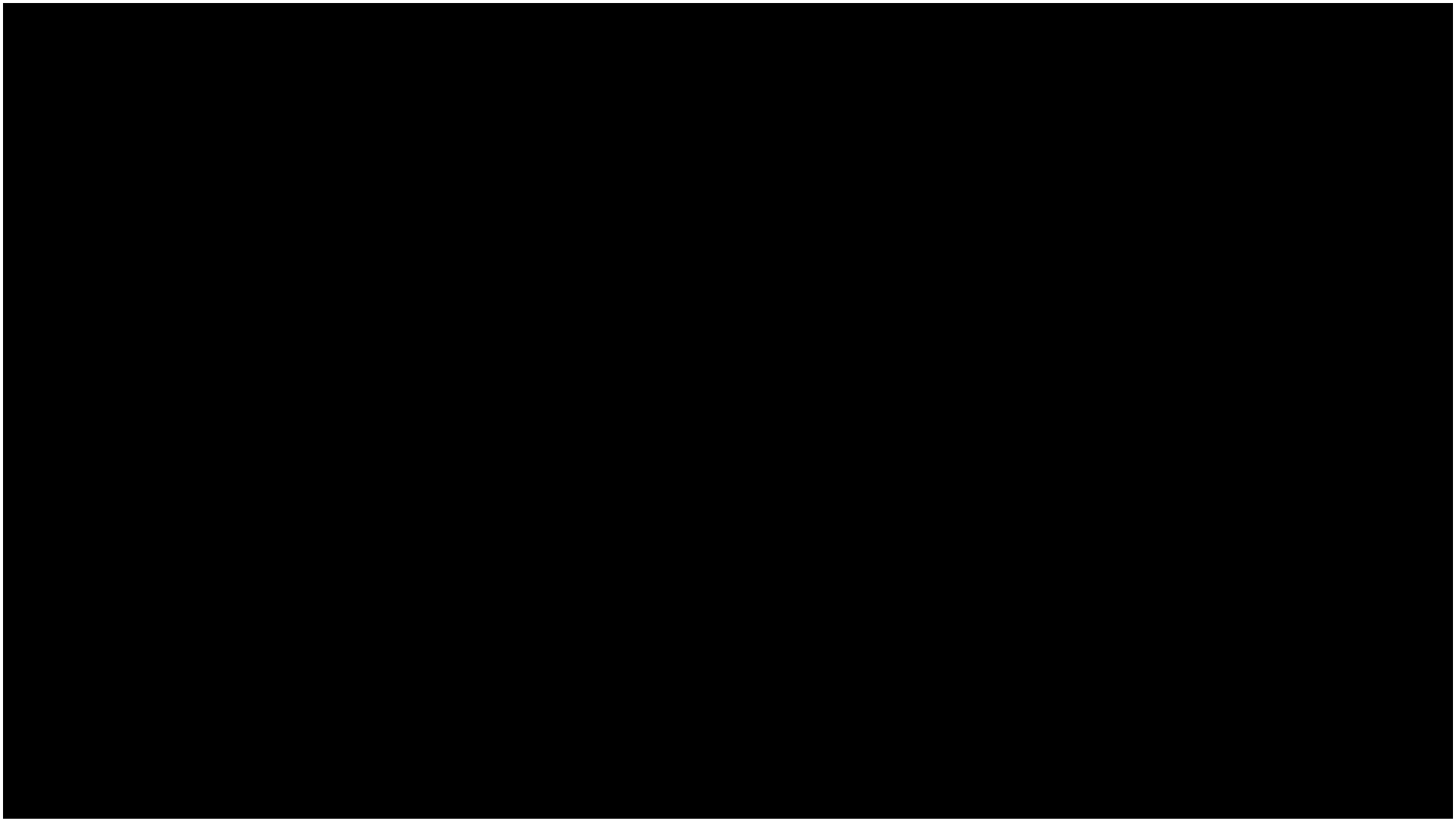

ssDNA–SSB^{AF488}
ssDNA–SSB
ssDNA–RecA^{f}
2 µm
Buffer flow
Tether

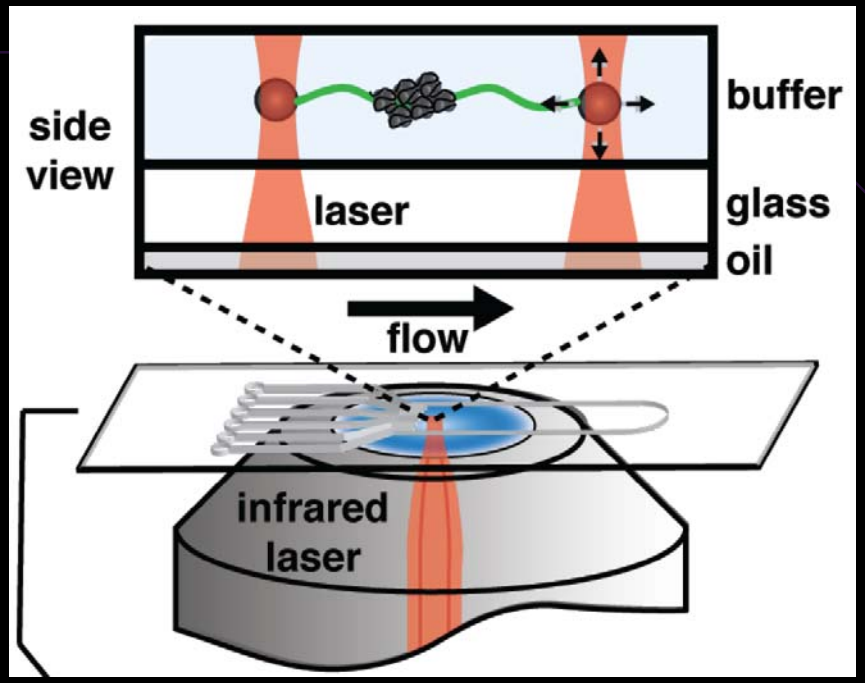

$k_i = 2k_e$
$k_i = k_e$
100 nM
200 nM
350 nM
Growth rate of internal clusters (k_i, nm min^{-1})
Growth rate of clusters near end (k_e, nm min^{-1})
0
20
40
60
80
100

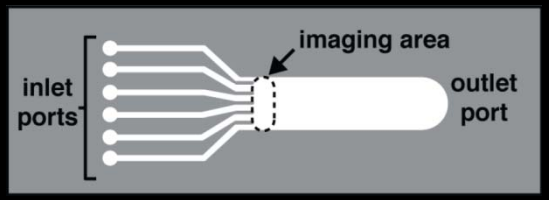

Time
SSB^AF488
1 μm
10′
RecA^Cy3
20′
27′
RecA^f
35′
43′
Flow
Nucleate with RecA^Cy3
Exchange RecA^Cy3 for RecA^f
Measure growth of RecA^f
3′ end
5′ end
Growth rate (nm min⁻¹)
80
60
40
20
0
5′ → 3′
3′ → 5′
Direction of growth

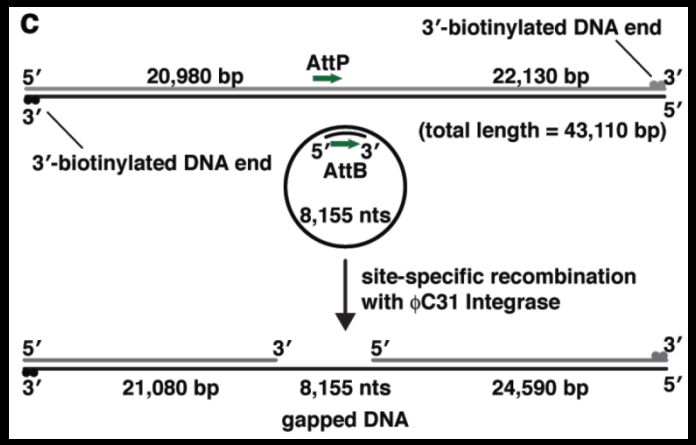

a
Nucleation rate (clusters 10k nt^{-1} min^{-1})
0.04
0.03
0.02
0.01
0.00
0
2
4
6
8
[Mg(OAc)$_2$] (mM)
23 °C
ATPγS
b
Growth rate (nm min^{-1})
80
60
40
20
0
0
2
4
6
8
[Mg(OAc)$_2$] (mM)
23 °C
ATPγS
c
Nucleation rate (clusters 10k nt^{-1} min^{-1})
0.10
0.08
0.06
0.04
0.02
0.00
ATPγS
dATP
ATP
23 °C
Nucleotide cofactor
d
Growth rate (nm min^{-1})
120
90
60
30
0
ATPγS
dATP
ATP
23 °C
Nucleotide cofactor
e
Nucleation rate (clusters 10k nt^{-1} min^{-1})
0.10
0.08
0.06
0.04
0.02
0.00
23 °C
ATPγS
6.0
6.5
7.0
7.5
8.0
8.5
pH
f
Growth rate (nm min^{-1})
120
90
60
30
0
23 °C
ATPγS
6.0
6.5
7.0
7.5
8.0
8.5
pH


g
Gapped DNA dumb-bell
dsDNA
dsDNA
SSB–ssDNA
Rotate 90°
Trap
0′
2′
4′
6′
8′
Stain
h
Lag time (min)
37 °C
ATPγS
RecA
+RecOR
+RecFOR
i
Growth rate (nm min−1)
37 °C
ATPγS
RecA
+RecOR
+RecFOR
j
1. SSB sliding or transient unwrapping
2. Dimerization and nucleation of RecA
3. Growth is enhanced by RecOR–SSB interactions
SSB–ssDNA blocks RecA
RecA
RecOR and RecFOR enhance nucleation




side
view
buffer
laser
glass
oil
flow
infrared
laser


imaging area
inlet
ports
outlet
port


C
3′-biotinylated DNA end
5′
20,980 bp
AttP
22,130 bp
3′
3′
5′
(total length = 43,110 bp)
3′-biotinylated DNA end
5′
3′
AttB
8,155 nts
site-specific recombination
with ϕC31 Integrase
5′
3′
5′
3′
3′
21,080 bp
8,155 nts
24,590 bp
5′
gapped DNA