Dynamics and selective remodeling of the DNA-binding domains of RPA

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Replication protein A (RPA) coordinates important DNA metabolic events by stabilizing single-stranded DNA (ssDNA) intermediates, activating the DNA-damage response and handing off ssDNA to the appropriate downstream players. Six DNA-binding domains (DBDs) in RPA promote high-affinity binding to ssDNA yet also allow RPA displacement by lower affinity proteins. We generated fluorescent versions of Saccharomyces cerevisiae RPA and visualized the conformational dynamics of individual DBDs in the context of the full-length protein. We show that both DBD-A and DBD-D rapidly bind to and dissociate from ssDNA while RPA remains bound to ssDNA. The recombination mediator protein Rad52 selectively modulates the dynamics of DBD-D. These findings reveal how RPA-interacting proteins with lower ssDNA binding affinities can access the occluded ssDNA and remodel individual DBDs to replace RPA.

In eukaryotic cells, RPA binds to transiently exposed ssDNA and serves as a hub protein to coordinate DNA replication, recombination, repair and telomere maintenance1–4. The cellular functions of RPA rely on its high ssDNA-binding affinity and its ability to physically interact with over two dozen DNA-processing enzymes and to correctly position these enzymes on complex DNA structures. The precise mechanisms by which RPA functions in many contexts and how RPA differentiates among multiple DNA metabolic events is a long-standing puzzle1–4. RPA is heterotrimeric, flexible and modular in structure. It is composed of three subunits, RPA70, RPA32 and RPA14 (Fig. 1a,b), which harbor six oligonucleotide/oligosaccharide-binding folds (labeled A through F). We refer to the DNA-binding oligonucleotide/oligosaccharide-binding folds as ‘DBDs’ (Fig. 1b). RPA binds to ssDNA with sub-nanomolar affinity but can be displaced by DNA-binding proteins with much lower DNA-binding affinity. Recent studies have suggested that the RPA-ssDNA complex is relatively dynamic1–4, possibly a dissociative mechanism where by not all DBDs are stably bound to the DNA, whereas microscopic dissociation of individual DBDs occurs.

In all existing models of RPA function, DBD-A and DBD-B are assigned as high-affinity DBDs. Purified DBD-A, DBD-B and DBD-A/DBD-B constructs bind ssDNA with equilibrium dissociation rate constant (Kd) values of 2 μM, 20 μM and 50 nM, respectively1–4. The trimerization core made up of DBD-C, DBD-D and DBD-E is considered to have a weaker ssDNA binding affinity (Kd > 5 μM)10. Additionally, mutational analysis of individual aromatic residues that interact with the ssDNA in either DBD-C or DBD-D show minimal perturbations in ssDNA binding affinity11,12. Paradoxically, in the crystal structure of the RPA-ssDNA complex (Fig. 1b), the ssDNA interactions of all four DBDs are similar, with DBD-C having more contacts with ssDNA bases than does DBD-A, DBD-B or DBD-D12. Thus, the exact nature of the contributions from each DBD to RPA function is probably complicated and may be influenced by the dynamics of DBD-ssDNA interactions.

Both the N terminus of RPA70 and the C terminus of RPA32 interact with distinct sets of RPA-interacting proteins (RIPs). During DNA processing, RIPs must displace RPA from ssDNA. Dissociation may be achieved by modulation of the DNA-binding activity of specific DBDs within RPA. In such a model, a protein that exchanges for RPA does not dissociate all DBDs at once but displaces individual DBDs after gaining access to DNA that is transiently exposed by dissociation of a DBD. Moreover, if the RPA-ssDNA complex were to be considered a sequential, linear assembly of DBDs, as seen in the crystal structure, then, depending on the DBD first displaced, a downstream DNA-binding protein could be positioned at the 5’ or 3’ end of the RPA-occluded ssDNA.

The recombination mediator Rad52 is one example of a RIP. It belongs to a group of proteins that orchestrate homologous recombination and homology-directed DNA repair. S. cerevisiae Rad52 regulates recombination by facilitating replacement of RPA on ssDNA with the Rad51 nucleoprotein filament, an active species in homology search and DNA strand exchange13–16. Nucleation of the Rad51 filament is a slow and tightly controlled process, as Rad51 fails to compete for binding to ssDNA with RPA17. Thus, Rad52 must physically interact with both RPA and Rad51 to promote Rad51 filament nucleation. The mechanism by which Rad52 loads Rad51 onto the ssDNA is unclear, except that Rad51 filament formation is simultaneous with displacement of RPA from ssDNA and probably proceeds through a Rad52–RPA–ssDNA intermediate18. Within this complex, Rad52 was shown to stabilize the RPA-ssDNA interaction18, further mystifying its assigned mechanism of action as a recombination mediator.

To determine how individual DBDs work in the context of the full-length protein and investigate how proteins such as Rad52 modulate RPA binding, we generated fluorescent forms of RPA containing a non-canonical amino acid that is labeled with the fluorescent dye MB543 in either DBD-A or DBD-D. When MB543 is positioned near the DNA-binding site, the fluorescence intensity of MB543 changes after binding to ssDNA occurs. Using direct measurements of full-length RPA carrying a fluorescently labeled DBD binding to and dissociating from ssDNA, we show that both DBD-A and DBD-D are highly dynamic, frequently binding to and dissociating

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Non-canonical amino acid–based fluorescent RPA molecules report on individual DBD dynamics. a, The residue numbers for the three RPA subunits and their respective DBDs (A–F) are denoted. The winged-helix (wh) domain in RPA32 and DBD-F in RPA70 mediate interactions with RIPs. The N terminus of RPA32 that is phosphorylated is shown in red. Crystal structures of the ordered domains are shown as surface representations with intervening disordered linkers as dotted lines (black). DBD-C, DBD-D and RPA14 interact to form the trimerization core. b, Crystal structure of the DBDs of Ustilago maydis RPA bound to ssDNA (PDB 4GNX). Residues T211 in DBD-A and W101 in DBD-D are sites where 4-azidophenylalanine (4-AZP) is incorporated (residue numbering in S. cerevisiae RPA). The bound ssDNA is shown as sticks (black). c, Coomassie and fluorescence imaging of RPA complexes labeled with MB543 at either DBD-A or DBD-D. Only the fluorescently labeled domains are visualized by fluorescence imaging, suggesting site-specific labeling of each domain. e, f, Binding of RPA–DBD–A\(^{MB543}\) and RPA–DBD–D\(^{MB543}\) to ssDNA was analyzed by monitoring the change in MB543 fluorescence. A robust change in fluorescence depicts engagement of specific DBDs onto ssDNA. Data were fit and analyzed as described in Methods. Values depicted in e, f represent the mean and s.e.m. from \(n = 3\) independent experiments. Uncropped gel images of c, d are shown in Supplementary Dataset 1.

Results

Direct readout of DBD dynamics using non-canonical amino acids and fluorescence. Directly monitoring the dynamics (binding, dissociation or remodeling) of a single enzyme in multiprotein reactions remains technically challenging. To decipher how the DBDs of RPA function in the context of the heterotrimeric RPA complex, we labeled DBD-A or DBD-D (in RPA70 or RPA32, respectively; Fig. 1c,d) of S. cerevisiae RPA\(^{19}\) with MB543, an environmentally sensitive fluorophore. Both fluorescently labeled RPA molecules are fully active for ssDNA binding with binding parameters and occluded binding site sizes typical of the wild-type RPA protein (Supplementary Note 1 and Supplementary Fig. 1). RPA labeled at domain A (RPA–DBD–A\(^{MB543}\)) or domain D (RPA–DBD–D\(^{MB543}\)) produces enhanced fluorescence after binding to ssDNA (Fig. 1c,d and Supplementary Fig. 2)\(^{19}\).

The binding of RPA to ssDNA is a paradigm for reactions in which multiple DNA-binding proteins function together on a single DNA template. Knowledge of where, how and when each protein gains access to the DNA in this multi-enzyme milieu is fundamental to deciphering when and how specific DNA repair/recombination processes are orchestrated. Site-specific labeling with MB543 allows us to monitor the dynamics of individual DBDs in the context of the full-length RPA heterotrimer and in multiprotein reactions. We measured the DNA-binding kinetics for RPA–DBD–A\(^{MB543}\) and RPA–DBD–D\(^{MB543}\), which provided direct readouts of each domain’s engagement with ssDNA in the context of full-length RPA. RPA–DBD–A\(^{MB543}\) or RPA–DBD–D\(^{MB543}\) was rapidly mixed with ssDNA ((dT)\(_{25}\)), and the change in fluorescence was measured (Fig. 1e,f). After binding to ssDNA, both RPA–DBD–A\(^{MB543}\) and RPA–DBD–D\(^{MB543}\) produce a change in fluorescence intensity. The data for RPA–DBD–A\(^{MB543}\) are best described by a two-step model (with observed rate constants \(k_{\text{obs,1}} = 30.6 \pm 9.8\) s\(^{-1}\) and \(k_{\text{obs,2}} = 10.3 \pm 9.8\) s\(^{-1}\)), whereas intensity changes associated with RPA–DBD–D\(^{MB543}\) best fit a single-step DNA-binding model (\(k_{\text{obs}} = 36.2 \pm 2.3\) s\(^{-1}\)). The first rate in both models is similar and reflects the initial interaction of RPA with ssDNA. The second step for RPA–DBD–A\(^{MB543}\) possibly reflects a rearrangement of DBD-A, as has been observed in structural studies\(^{20,21}\).
To probe the nature of these differences further, we performed these binding experiments as a function of increasing DNA concentration using (dT)\textsubscript{35}, which provides enough space for engagement of all the DBDs of RPA (Fig. 2). While measurements of RPA–ssDNA interaction footprints under our buffer conditions yield occluded site sizes of ~20 nucleotides (nt) per RPA (Supplementary Fig. 1h), the modularity of the DBDs have been shown to produce occluded site sizes between 18 nt and 28 nt (ref. 22).

The observed rate for the first association step for both RPA–DBD-A MB543 and RPA–DBD-D\textsubscript{MB543} increases as a function of DNA concentration, yielding bimolecular binding rate constant (\(k\textsubscript{obs,1}\)) values ((1.1 \pm 0.6) \times 10^8 \text{M}^{-1}\text{s}^{-1} and (2.1 \pm 0.4) \times 10^8 \text{M}^{-1}\text{s}^{-1}, respectively; Fig. 2a–c, f–h). The second step, observed only for RPA–DBD-A\textsubscript{MB543}, is not linear (Fig. 2c). This is consistent with a conformational rearrangement of DBD-A after the complex with ssDNA has been established and depends on the protein-to-DNA ratio in the reaction. Under conditions in which RPA is present in excess over ssDNA, we clearly observe biphasic binding and dissociation/rearrangement phases for RPA–DBD-D\textsubscript{MB543} (orange and pink traces in Fig. 2b) but not for RPA–DBD-D\textsubscript{MB543} (Fig. 2g). These data suggest that the dynamics of individual DBDs are not identical, possibly reflecting different functional roles.

FRET analysis confirms primary assessments of DBD–ssDNA dynamics. In the experiments described above, the change in fluorescence intensity arises from local environmental changes of the fluorophore after binding to the ssDNA. We suggest that changes in the MB543 fluorescence reflect changes in the electrostatic environment of the dye (Supplementary Fig. 2). To reaffirm that the accuracy of the dynamics we measured for each fluorescent DBD reflects ssDNA interactions, we used Förster resonance energy transfer (FRET) to capture DBD–ssDNA-binding kinetics. RPA binds to ssDNA with specific polarity where DBD-A is positioned closer to the 5’ end of the ssDNA\textsuperscript{12,23}. Similar to the MB543-labeled proteins, we generated Cy5-labeled RPAs in which either DBD-A or DBD-D was labeled with Cy5. We next performed FRET experiments with either 5’- or 3’-Cy3-end-labeled DNA [(dT)\textsubscript{15}]. On 5’-Cy3 DNA, a high FRET signal is observed for RPA–DBD-A\textsuperscript{Cy5} and a medium FRET state is captured for RPA–DBD-D\textsuperscript{Cy5} (Supplementary Fig. 3a,b). In the corollary experiment with 3’-Cy3 DNA, a low FRET state for RPA–DBD-A\textsuperscript{Cy5} and a high FRET state for RPA–DBD-D\textsuperscript{Cy5} are observed (Supplementary Fig. 3c,d). These experiments are consistent with the expected 5’–to–3’ polarity of RPA binding. Strikingly, the observed rate for the appearance of the RPA–DBD-A\textsuperscript{Cy5} high FRET state (36 \pm 2 s\textsuperscript{-1}; Supplementary Fig. 3d) agrees with the rate change in fluorescent intensity of RPA–DBD-D\textsuperscript{Cy5} after binding to ssDNA (36.2 \pm 2 s\textsuperscript{-1}; Fig. 11). Similarly, the observed rate of 21 \pm 1 s\textsuperscript{-1} for the appearance of the RPA–DBD-A\textsuperscript{Cy5} high FRET state (Supplementary Fig. 3b) is probably a composite of the two observed phases captured in fluorescence-intensity changes of the RPA–DBD-A\textsubscript{MB543}–ssDNA complex (\(k\textsubscript{obs,1} = 30 \pm 1 \text{s}^{-1}\) and \(k\textsubscript{obs,2} = 104 \pm 2\); Fig. 1c). The FRET data affirm that the ssDNA-binding responsive fluorescence intensity enhancements reflect specific DBD–ssDNA interactions.

Differential effects of ssDNA length on DBD conformations. Since each DBD has varying footprints on ssDNA\textsuperscript{12}, we measured the DBD dynamics as a function of ssDNA length and found that the \(k\textsubscript{obs,1}\) increases as a function of ssDNA length for RPA–DBD–A\textsubscript{MB543} (Fig. 2d, e), whereas the same parameter saturated for RPA–DBD–D\textsubscript{MB543} at ~20 nt (Fig. 2i, j). On shorter DNA lengths, both binding and dissociation phases are clearly observed for RPA–DBD–A\textsubscript{MB543} (Fig. 2d, (dT)\textsubscript{15} and (dT)\textsubscript{20} traces); however, only a single binding phase for RPA–DBD–D\textsubscript{MB543} is observed with all ssDNA lengths (Fig. 2i). Since ssDNA and RPA are in molar equivalents in these experiments, the dissociation from shorter DNA probably occurs from intrasubunit competition among the four DBDs of RPA. DBD-F, DBD-A and DBD-B are considered the conformationally flexible half\textsuperscript{24}. In contrast, DBD-C, DBD-D and DBD-E constitutively interact to form the trimerization core and are more conformationally rigid. We considered the possibility that the trimerization core might be overcoming the more dynamic DBD-A (and possibly...
Changes in fluorescence could be attributed to single RPA molecules with the buffer (Fig. 3a,b). The final step ensured that the observed fluorescence states during the last 90 s of the experiment is due to the dissociation and rebinding of RPA, we substituted the buffer wash with a buffer supplemented with high concentration of ssDNA. In the absence of additional RPA in the solution, the ssDNA competitor cannot strip the bound RPA from the DNA but can sequester all dissociated RPA molecules. As expected, the addition of ssDNA to the reaction chamber had no effect on the RPA fluorescence states (Supplementary Fig. 7 and Supplementary Table 2).

We ruled out the possibility of photophysical effects as the source of the MB543 fluorescence states by repeating the experiments at three different powers of the excitation laser, as true conformational states should not display any trend in power dependence\(^{20,28}\). The data, summarized in Supplementary Fig. 6c,d and Supplementary Table 3, show no change in the laser power dependence for any of the four states of RPA–DBD-D\(^{MB543}\), thus confirming that this is the case. Additionally, the absence of distinct fluorescence states for RPA alone, with the exception of the infrequent visitation of the dimmest state, suggests that the observed states are induced by the interaction with ssDNA (Supplementary Fig. 6a,b). The four fluorescence states and their dwell times were consistent between independent experiments, suggesting that the normalization scheme we developed yields reproducible results (Fig. 3l,o,p). For both RPA–DBD-A\(^{MB543}\) and RPA–DBD-D\(^{MB543}\), states 1 and 4 were the most stable, with average dwell times around 1 s, compared to states 2 and 3, whose average dwell times were between 300 ms and 500 ms. As evident from the representative trajectories (Fig. 3c,d and Supplementary Dataset 2 and 3), RPA spends substantial periods of time in states in which DBD-A or DBD-D is not fully engaged, providing a window of binding opportunity for proteins of lower affinity.

In addition, we found that the collective DNA-binding affinities of all DBDs produce stable RPA–ssDNA complexes. DBD-A and DBD-B have been canonically assigned as being responsible for high-affinity DNA binding of the RPA complex. By carrying out single-molecule experiments with RPA–FAB–A\(^{MB543}\), we found that it forms a less-stable complex on ssDNA and readily dissociates (Supplementary Fig. 4e–h). These findings agree with the results from the bulk stopped-flow experiments (Supplementary Fig. 8 and Supplementary Note 2). Fluorescence trajectories recorded for RPA–FAB–A\(^{MB543}\) further confirms that the four states observed for the full-length RPA are not photophysical states inherent to the MB543 dye.

Rad52 selectively modulates DBD-D dynamics. To determine the mechanism by which the recombination mediator Rad52 remodels ebFRET\(^{27,28}\). The number of trajectories and states in each experiment are summarized in Supplementary Table 1 and Supplementary Dataset 4. This analysis revealed that the fluorescence derived from both proteins best fit a four-state model, with state 1 corresponding to very low fluorescence and states 2–4 corresponding to increasing fluorescence enhancement (Fig. 3c,d). Segments of the trajectories between 120 s and 210 s, which can be attributed to the dynamics of a single bound RPA, were used in the quantification of the lifetimes and visitation frequencies for all states.

We attribute state 4 in each case to the RPA conformation in which the labeled domain is probably fully engaging the ssDNA, because in bulk experiments we observed an ssDNA-dependent fluorescence increase in the full-length RPA carrying the fluorescent probe and RPA–FAB–A\(^{MB543}\) (containing only DBD-A and DBD-B). To rule out the possibility that the lowest fluorescence state (state 1) followed by the reappearance of the fluorescence during the last 90 s of the experiment is due to the dissociation and rebinding of RPA, we substituted the buffer wash with a buffer supplemented with high concentration of ssDNA. In the absence of additional RPA in the solution, the ssDNA competitor cannot strip the bound RPA from the DNA but can sequester all dissociated RPA molecules. As expected, the addition of ssDNA to the reaction chamber had no effect on the RPA fluorescence states (Supplementary Fig. 7 and Supplementary Table 2).

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Rad52 selectively modulates DBD-D dynamics. To determine the mechanism by which the recombination mediator Rad52 remodels
Single-molecule analysis quantifies the conformational dynamics of DBDs and the effect of the recombination mediator Rad52.

Experimental scheme for visualizing conformational dynamics of DBD-A and DBD-D. Binding of fluorescently labeled RPA to a surface-tethered ssDNA (purple line) brings the MB543 fluorophore within the evanescent field of TIRFM. NA, NeutrAvidin; b, biotin.

**Representative fluorescence trajectories for individual RPA–DBD-A MB543 and RPA–DBD-D MB543 molecules (purple lines and green lines, respectively). Black lines are the result of ebFRET fitting.** Additional examples of representative trajectories are presented in Supplementary Datasets 5 and 6.

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**Representative fluorescence trajectories depicting conformational dynamics of DBD-A and DBD-D.** Binding of fluorescently labeled RPA to a surface-tethered ssDNA (purple line) brings the MB543 fluorophore within the evanescent field of TIRFM. NA, NeutrAvidin; b, biotin.

**Experimental scheme for visualizing the effect of Rad52 on the conformational dynamics of DBD-A and DBD-D.**

**Representative fluorescence trajectories for individual RPA–DBD-AMB543 and RPA–DBD-DMB543 molecules (purple lines and green lines, respectively). Black lines are the result of ebFRET fitting.** Additional examples of representative trajectories are presented in Supplementary Datasets 5 and 6.

**Representative fluorescence trajectories depicting conformational dynamics of the individual RPA–DBD-A MB543 and RPA–DBD-D MB543 molecules after the addition of Rad52.** Additional trajectories are shown in Supplementary Datasets 5 and 6.

**Dwell-time histograms for the four fluorescent states obtained by the ebFRET fitting of RPA–DBD-A MB543 and RPA–DBD-D MB543 trajectories from three independent experiments after buffer wash (i) and Rad52 wash (j). Before analysis, the trajectories were cut from 120 s to 210 s. Solid lines represent the single exponential fit.** The data are summarized in Supplementary Table 1.

**The two-way analysis of variance (ANOVA) suggests no significant differences between the dwell-time distributions.** The same analysis was carried out for RPA–DBD-D MB543. Only three fluorescence states were detected in the presence of Rad52. NP, not present. Statistical analysis is performed by ANOVA (***, P=0.0001 and ****, P<0.0001).
the RPA–ssDNA complex, RPA–DBD-D<sub>AMB543</sub> or RPA–DBD-D<sub>DMS143</sub> bound to the surface-tethered ssDNA in the smTIRFM experiments were challenged with Rad52 (Fig. 3e,f and Supplementary Datasets 5 and 6). The final 90-s portions of the resulting trajectories were normalized and globally analyzed using ebFRET. Dwell times for each state were binned and fit to an exponential decay (Fig. 3i, j,m,n, Supplementary Dataset 4 and Supplementary Table 1). RPA–DBD-D<sub>DMS143</sub> trajectories after buffer wash or after Rad52 addition fit best to a four-state model with the same distribution of states and the same dwell times (Fig. 3i–l). The trajectories collected for RPA–DBD-D<sub>DMS143</sub> after Rad52 addition instead best fit a three-state model (Fig. 3m–p). Attempts to fit these trajectories with a four-state model resulted in overfitting and overlapping states. Intensities of the three states of RPA–DBD-D<sub>DMS143</sub> after Rad52 addition correspond to the three lowest states seen after the buffer wash, with the highest state being absent when Rad52 was present (Fig. 3h, blue shaded area). With RPA–DBD-D<sub>DMS143</sub>, state 4 is lost after Rad52 addition and state 3 occupancy decreases, whereas state 1 and state 2 occupancy increases (Fig. 3o). This suggests that Rad52 selectively modulates the conformational dynamics of the RPA–ssDNA complex, reducing the engagement of DBD-D with ssDNA and providing access to the 3′ end of the occluded ssDNA.

Formation of the RPA–ssDNA–Rad52 complex depends on physical interaction between RPA and Rad52, which is mediated by ssDNA and is confined to the middle region of the Rad52 C-terminal domain<sup>34,46</sup>. To test whether the modulation of DBD-D by Rad52 depends on interaction between the two proteins, we used human Rad52, which resembles the yeast protein in all its activities but does not interact with yeast RPA<sup>34</sup>. We found that human Rad52 does not alter the four states of the interaction of RPA–DBD-D<sub>DMS143</sub> with DNA (Supplementary Fig. 9a–d). Thus, the modulation of state 4 is specific for yeast Rad52 and therefore requires Rad52–RPA interaction. To highlight the importance of the interaction between Rad52 and ssDNA for modulating the RPA conformational dynamics, we performed an experiment in the presence of a Rad52 inhibitor, epigallocatechin (EGC) (Supplementary Fig. 9e–f). EGC inhibits the DNA-binding activity of human RAD52<sup>32</sup> and yeast RAD52, but not that of RPA (Supplementary Fig. 9g–k and Supplementary Table 4). These data suggest that the loss of state 4 also depends on the DNA-binding activity of Rad52.

**Discussion**

Prior RPA binding models have been based on analysis of subcomplexes or mutations. In contrast, we have analyzed the dynamics of individual domains in the context of the full RPA–ssDNA complex. This analysis demonstrates that rather than being composed of ‘high’ and ‘low’ affinity domains, all DBDs transiently engage ssDNA with high affinity, but with differential dynamics. We also observe interplay between the flexible half of RPA (DBD-F, DBD-A and DBD-B) and the trimerization core. Thus, the RPA–ssDNA complex consists of an ensemble of domains that dynamically interact with ssDNA. This suggests that integration of these interactions results in an overall high affinity for ssDNA while facilitating diverse functions of RPA. Our data also suggest that the assembly of DBDs on ssDNA is not sequential but is instead the result of dynamic, independent interactions between connected DBDs and DNA.

RPA forms a complex with the recombination mediator Rad52<sup>32,36</sup>. RPA is in dynamic equilibrium on ssDNA, and Rad52 has been shown to increase the residence time of RPA on ssDNA<sup>36</sup>. The formation of ‘early Rad52-bound’ RPA and that of ‘late Rad52-bound’ RPA are proposed to play distinct roles during Rad51 filament formation and second-strand capture during homologous recombination<sup>14</sup>. The ability to observe the individual RPA DBDs binding to, and dissociating from, the ssDNA in real time permits a mechanistic description of RPA–ssDNA–Rad52 interactions. The RPA heterotrimer and the heptameric Rad52 ring have similar ssDNA-binding sites. Each Rad52 monomer contains an RPA-binding site, and RPA has two Rad52-binding sites per heterotrimer. Rad52 is believed to interact with the ssDNA backbone, while the DBDs of RPA, especially within the trimerization core, engage the bases. Our results show that stabilization of RPA by Rad52 is a result of both their physical interactions and their individual interactions with DNA. We therefore envision a ternary complex in which Rad52 and RPA interact with one another while both proteins are simultaneously bound to ssDNA. Selective modulation of DBD-D ssDNA engagement by Rad52 provides space for Rad52 to interact with ssDNA and stabilize the ternary complex, which then makes more contacts with the ssDNA than RPA does on its own. By redistributing the ssDNA between RPA and Rad52, and by reducing the contacts between RPA and ssDNA, such selective remodeling by Rad52 may provide the Rad51 recombinase access to the 3′ end of RPA-occluded ssDNA while maintaining its interaction with RPA (Fig. 4). Each Rad51 monomer binds to one monomer of Rad52 and to three nucleotides of ssDNA. Six Rad51 monomers are required to achieve a stable nucleation cluster<sup>46</sup>, which amounts to 18 nucleotides of open ssDNA. This cannot be achieved without the help of a recombination mediator. When Rad52 binds to ssDNA-bound RPA, it modifies the dynamics of the DBD-D engagement. This provides a stretch of ssDNA of sufficient length to initiate Rad51 filament nucleation. We predict that recombination mediators in other species, including human BRCA2, may operate by a similar mechanism. The details of this mechanism, however, will depend on the intrinsic differences in nucleoprotein filament formation by human RAD51, which nucleates on ssDNA through dynamic association of
RAD51 dimers and grows from heterogeneous nuclei ranging in size from dimers to oligomers.

The myriad roles of RPA in DNA replication, repair and recombination are also a paradigm for reactions in which multiple DNA-binding enzymes function together on a single DNA template. Knowledge of where, how and when each enzyme gains access to DNA in this multi-enzyme milieu is fundamental to deciphering when and how specific DNA repair and recombination processes are established and used. RPA–ssDNA complexes serve as binding targets for recruitment of the appropriate enzymes during various DNA metabolic processes, and physical interactions between RPA and more than two dozen enzymes have been identified. After recruitment, the bound ssDNA must be handed over from RPA or remodelled in such a way that the DNA is accessible to the incoming enzyme while RPA remains at the site. Microscopic binding and dissociation of the RPA DBDs is likely to enable the persistent residence of RPA at the site of repair as well as its ability to coordinate access to the DNA by helicases and nucleases. Such a mechanism might also be applicable to RPA-like proteins that also carry a multiioligonucleotide/oligosaccharide-binding fold architecture, such as the CST complex associated with telomerase.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41594-018-0181-y.

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Author contributions
N.P., C.C.C., E.I.C., N.J. and E.A.T. performed experiments. J.T. and S.M.A.T. developed the MatLab scripts for data analysis. E.A., M.S., M.S.W., N.P. and C.C.C. conceived and designed the experiments and wrote the manuscript.

Competing interests
The authors declare no competing interests.

Additional information
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Plasmids and protein expression. Plasmids used in this study are detailed in Supplementary Note 3. 

Methods

Chemicals and reagents. 4-Azidophenylalanine (4-AZP) was prepared from Fmoc-4-amino-phenylalanine (Angene Inc.) as described. All fluorophores used to generate fluorescently labeled proteins were purchased from Click Chemistry Tools. Cy3-labeled, biotinylated and unmodified oligonucleotides were purchased form Integrated DNA Technologies.

Supplementary Note 3. 

RPA variants were separated from excess dye using a Biogel-P4 gel filtration on a rocker with a 1.5-fold molar excess (150,000 M−1 cm−1 for DBCO-Cy3, and at 650 nm, with ε = 250,000 M−1 cm−1 for DBCO-Cy5 fluorophores. We obtained 45 ± 17 and 40 ± 25% labeling efficiencies for the RPA–DBD-AMB543 and RPA–DBD-DCy5, respectively.

Fluorescence measurements. Fluorescence spectra were obtained using a Pti QM40 instrument (Horiba Scientific). For RPA–DBD-AMB543, RPA–DBD-DCy5 and RPA–DBD-Acy5, slit widths were set at 1.25 nm for excitation and 3 nm for emission. For RPA–DBD-D30 and RPA–DBD-Acy5, slit widths were set at 0.5 nm for excitation and 2 nm for emission. For RPA–DBD-Acy5, 2 ml of 50 nM RPA–DBD-Acy5 in reaction buffer (30 mM Hepes, pH 7.8, 100 mM KC1, 5 mM MgCl2, 1 mM β-mercaptoethanol and 6% v/v glycerol) was added to a quartz cuvette, and the spectra were collected in the absence or presence of 50 nM ssDNA (dT)50 or double-stranded plasmid DNA (100 nM nucleotides) with constant stirring. Samples were excited at 355 nm, and emission spectra (555–600 nm) were recorded. A similar experimental setup was used to obtain fluorescence spectra for 50 nM RPA–FABMB543 after the addition of 100 nM (dT)30. For RPA–DBD-D30 and RPA–DBD-Acy5, 2 ml of 100 nM protein was used and 100 nM of (dT)30 was added with constant stirring. The samples were excited at 559 nm, and emission spectra (539–579 nm) were recorded. Similarly, for RPA–DBD-Acy5 and RPA–DBD-D30, 2 ml of 100 nM proteins were used and 100 nM of (dT)30 was added to the reaction. Samples were excited at 690 nm, and emission spectra (640–700 nm) were recorded. All experiments were performed at 25 °C. The total number of nucleotides occupied (site size) by wild-type (wt) RPA and its variants (RPA-wt, RPA–DBD-AMB543 and RPA–DBD-DCy5) in reaction buffer was determined as previously described using poly(dT) ssDNA.

DNA binding. The DNA-binding activity of unlabeled and fluorescent RPA was measured using electromobility band-shift analysis. 50 nM of [32P]-labeled (dT)30 oligonucleotide was incubated with increasing concentrations of RPA-wt or RPA–DBD-AMB543 or RPA–DBD-D30 (0–1 μM) in reaction buffer for 10 min at 4 °C. 1 ml of ssDNA loading dye (30% w/v glycerol and 0.2% w/v bromophenol blue in 10 mM borate-EDTA (TBE) buffer) was added to the sample, and samples were resolved using an 8% TBE–acrylamide gel (110 V, 25 °C). Gels were exposed overnight onto a phosphorimaging screen and scanned using a STORM scanner (GE Healthcare). Bound and unbound DNA signals were quantitated using the equation:

\[
\Delta F = A_1 \left(1-e^{-k_1 t}\right) + A_2 \left(1-e^{-k_2 t}\right) + k_t
\]

where ΔF is the change in fluorescence signal, A1 and A2 are the amplitude of fluorescence change, k1 and k2 are the observed rate constants and t is time. For RPA–DBD-D30 and RPA–FABMB543, the data were well described by a single-step binding model defined phenomenologically by a single exponential plus linear equation:

\[
\Delta F = A_1 \left(1-e^{-k_1 t}\right) + k_t
\]

To measure the facilitated exchange of RPA–DBD-Acy5 and RPA–DBD-DCy5 for DNA binding to ssDNA, 200 nM RPA–DBD-Acy5 in one syringe was rapidly mixed with increasing concentrations (50–400 nM) of (dT)30, oligonucleotide from a second syringe, and the change in fluorescence of RPA–DBD-Acy5 was monitored. All the data obtained were analyzed using equation (2) to obtain observed rate constants. Then, kobs,1 was plotted against the concentration of (dT)30, and a linear fit was used to generate a rate for the binding of DBD-A to ssDNA. Similarly, to measure the binding of DBD-D to ssDNA, 200 nM RPA–DBD-D30 in one syringe was rapidly mixed with increasing concentrations (50–400 nM) of (dT)30, oligonucleotide from a second syringe, and the change in fluorescence of RPA–DBD-D30 was monitored. Finally, to obtain the rate of association of EAB binding to ssDNA, 300 nM FABMB543 in one syringe was rapidly mixed with increasing concentrations (100–1000 nM) of (dT)30 oligonucleotide from a second syringe, and the change in fluorescence of FABMB543 was monitored. For RPA–DBD-D30 and RPA–FABMB543, all the data obtained were analyzed using equation (3) to obtain the observed rate constants. Then, kobs,1 was plotted against the concentration of (dT)30 and against that of (dT)30 for RPA–DBD-D30 and RPA–FABMB543, respectively. A linear fit was used to generate a rate for the binding of DBD-D and FAB to ssDNA.

RPA–DBD-Acy5 and RPA–DBD-D30 length-dependent association kinetics. To measure the oligonucleotide length-dependent rate of association for DBD-A, 200 nM RPA–DBD-Acy5 was rapidly mixed with 200 nM (dT)50, (dT)100, (dT)200, (dT)500 and (dT)1000, and the change in fluorescence was monitored. All the data obtained were analyzed using equation (2) to obtain the observed rates. Then, kobs,1 was plotted against (dT)n, to determine the rate for the oligonucleotide-length-dependent association of DBD-A. Using the same concentration of reactants, similar experiments were performed with RPA–DBD-D30 and RPA–FABMB543 to measure the oligonucleotide-length-dependent rate of association for DBD-D. All the data obtained were analyzed using equation (3) to obtain the observed rates. Then, kobs,1 was plotted against (dT)n to determine the rate for the oligonucleotide-length-dependent association of FAB and DBD-D.

RPA–DBD-Acy5 and RPA–DBD-D30 polarity for DNA binding. For all FRET experiments, samples (Cy5) were excited at 555 nm, and Cy5 fluorescence emission was monitored with a 645 nm long-pass emission filter. 5'- or 3'-Cy5-labeled DNA (100 nM) was mixed with the appropriate Cy5-labeled RPA protein, and the change in fluorescence was captured. Data were fit to equation (3) to observe binding rates constant for the resulting change in fluorescence.

Tryptophan binding kinetics. Changes in tryptophan fluorescence were monitored by exciting the samples at 290 nm, and emission was measured using a 350 nm cut-off filter. For the FRET experiments, the Cy5-labeled RPA and Cy3-labeled DNA samples were mixed and excited at 555 nm, and changes in Cy5 fluorescence were monitored with a 645 nm cut-off emission filter.
RPA–DBD-D<sup>35ssDNA</sup>) was rapidly mixed with increasing concentrations (30–400 nM) of (DT)_<sub>10</sub> oligonucleotide, and the change in Trp fluorescence was monitored by exciting the sample at 290 nm and measuring emission with a 350-nm cut-off filter. Data were fitted using equation (3), and k<sub>off</sub>, was plotted against the concentration of (DT)_<sub>10</sub>. A linear fit was used to obtain the observed rate constants for RPA–DNA binding.

**Single-molecule imaging.** A custom-built prism-type total internal reflection microscope was used to image single-molecule events. A diode-pumped solid-state green laser (532 nm, Coherent) was used to excite the MB543 dye. The laser power output was set to 45 mW at the entrance to the microscope stage, unless otherwise noted. A dual-band-pass filter (Semrock; FF01-577/700) was used to filter out scattered light in the emission optical path, and the MB543 fluorescence was collected using a Chroma ET605/70m filter. Videos were recorded using an electronic multiphoton charge-coupled device camera (Andor; DU-897-E-CSD-#BV) at 100-ms time resolution. Background was set to 400, correction was set to 1,200 and gain was set to 295 for all videos recorded.

**FRET-based assays for Rad52 inhibition.** Epigallocatechin (EGC, Sigma-Aldrich) has been previously characterized as an inhibitor of human RAD52–ssDNA binding<sup>35</sup>. To verify that EGC also inhibits the ssDNA binding of human RAD52, we carried out FRET-based inhibitor assays as described<sup>11</sup>. A Cary Eclipse spectrophotometer was used to monitor the Cy3 and Cy5 emission simultaneously. Cy3 was excited at 530 nm, and emission was collected at 565 nm with slit widths of 10 nm. Cy5 was excited through FRET transfer with Cy3, and emission was collected at 660 nm with a slit width of 10 nm. Experiments were performed at 37°C in a buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 100 nM NaCl, 1 mM DTT and 0.1 mg ml<sup>−1</sup> BSA. EGC’s inhibition of Rad52–ssDNA binding was tested by pre-mixing 100 nM Rad52 and 10 mM Cy3–dT30-Cy5 ssDNA to form a stoichiometric complex, which corresponds to ssDNA fully wrapped around the oligomeric Rad52 ring and, correspondingly, the high FRET state<sup>11</sup>, and titrating increasing concentrations of EGC. Experiments were carried out in triplicate, and FRET signal and median inhibitory concentration were calculated as previously described<sup>35</sup>. EGC’s inhibition of RPA–ssDNA binding was tested by pre-mixing 10 nM RPA and 100 nM Cy3–dT30-Cy5 ssDNA to form a stoichiometric complex in which the DNA is straightened to its contour length<sup>41</sup>, and titrating increasing concentrations of EGC. Experiments were carried out in triplicate, and the FRET signal was calculated as described above.

**Single-molecule cell assembly.** Slides were washed, coated and flow cell assembled as described previously<sup>35</sup>. Reaction buffer containing 50 mM Tris–HCl, pH 7.5, 5 mM MgCl<sub>2</sub>, 100 nM NaCl, 1 mM DTT and 0.1 mg ml<sup>−1</sup> BSA and 0.8% glucose in Trolox was used for all single-molecule experiments. 12 mM Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid, Sigma-Aldrich; 238313-1G) was dissolved in 12 mM NaOH and was rotated under a fluorescent light (Sylvaia FM13W/835) for 3 days or until the absorbance at 400 nm was approximately 0.119. Assembled flow cells were first rinsed with T50 (10 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, 50 mM NaCl and 0.1 mg ml<sup>−1</sup> BSA). Increasing concentrations of E. coli cell were incubated for 3 min with 100 pM biotinylated 66-nt oligonucleotide (5′-CTC AAG CCA TCC GCA ACG TTT TTT TTT TTT TTT TTT TTT TTG GAA TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GAA ACA ACG GCC TCC TCA; IDT) in reaction buffer, instead of reaction buffer, and then rinsed with reaction buffer, 2.100 frames, 210-s video were recorded with 100 pM RPA–DBD-A<sup>35ssDNA</sup> or RPA–DBD-D<sup>35ssDNA</sup> added after the first 300 frames. At 1,200 frames, free RPA was either washed away with reaction buffer or replaced with 700 nM RPA. Experiments with RPA–FAB-A<sup>35ssDNA</sup> were carried out as described above, except 1 nM RPA–FAB-A<sup>35ssDNA</sup> was added at 300 frames with no wash. The experiment with human RAD52 was carried out as described, adding 700 pM human RAD52 instead of S. cerevisiae Rad52. EGC inhibitor single-molecule experiments were carried out as for the addition of Rad52 to RPA–DBD-D<sup>35ssDNA</sup> described above, with the addition of 10 nECG premixed with Rad52 in reaction buffer. To challenge RPA binding to ssDNA, experiments were performed with 1 nM of a 42-nt unlabeled ssDNA oligonucleotide (5′-TTT TTT TTT TTT TTT TTT TTG GAA TTA AGC TCT AAG CCA TCC-3′) added to the reaction buffer in the wash step. Experiments were recorded, except otherwise, the excitation laser power was set to 45 mW at the entrance to the microscope stage. Laser power experiments were performed as described above, but at 36 and 27 mW, and results were then compared to previous experiments carried out at maximum laser power (45 mW) of the green laser (532 nm).

**Surface-tethered RPA.** RPA–DBD-D<sup>35ssDNA</sup> contains a polyhistidine tag on the C terminus of RPA32, which was used to tether the protein to the slide surface. The slide was first incubated with NeutriAvidin and rinsed and was then incubated with Biotin-X-NTA buffer (50 mM Tris, pH 7.5, 50 mM NaCl and 20 mM Biotin-X-NTA, Sigma-Aldrich) for 10 min. The same reaction buffer was used, with the addition of 5 µM NiSO<sub>4</sub>. The chamber was rinsed with reaction buffer, followed by the addition of 500 pM RPA–DBD-D<sup>35ssDNA</sup>, which was incubated in the chamber for 5 min. The chamber was then rinsed with reaction buffer. A 2.100-frame, 210-s video was recorded.

**Single-molecule data analysis.** An IDL script was used to extract fluorescence intensity trajectories (changes in the fluorescence over time in a particular location on the flow cell) from each video (available upon request). Only those trajectories that show the appearance of the fluorescence signal between 30 s and 120 s (indicated as ON in Figs. 3c,d) were selected for analysis. Traces were viewed using a Matlab script and were selected using the following three criteria: at least two transitions, signal-to-noise ratio > 4 (raw signal), and no signal before addition of a fluorophore. Traces that did not meet these criteria were discarded.

The selected traces were then saved individually and processed for analysis by ebFRET. Since ebFRET was developed to analyze two-color trajectories, where recorded donor and acceptor fluorescence is recalculated into FRET efficiency, which is between 0 and 1, our single-color trajectories required normalization before analysis. To prepare fluorescence trajectories for the analysis by ebFRET, we used emulateFRET, a program that normalizes intensity in all experimental trajectories to be within the range of 0 to 1 (Supplementary Fig. 5). Fluorescent trajectories were imported into emulateFRET in batches and analyzed as a group. A percentile calculation determined a threshold for the maximum intensity in the group while excluding outliers. For each trajectory, the fluorescence in each point was then divided by this maximum intensity. This all traces to the range from 0 to 1. A weighted moving-average algorithm (of width 5) removed a small trailing tail of intensity. For each trajectory, a histogram of the values in the trace was produced (a histogram of fluorescence intensity values binned in intervals of 5 units was shown on the right of the raw trajectory in a light green color, and a brighter green histogram with overplotting Gaussian curve represented the baseline values for the first 30 s before the addition of fluorescent RPA). This histogram showed a characteristic peak surrounding the values that comprise the baseline of that trajectory. The shape of this peak on the histogram was fit to a Gaussian distribution. From this Gaussian distribution, the new baseline was determined and was placed 2 s.d. above the mean of this peak. All values below this baseline were reassigned to surround the new baseline value. This suppressed the ebFRET program’s tendency to assign multiple states to the typically over-represented baseline signal. The edited trajectories were given a dummy ‘donor’ trace, such calculated that the FRET signal of each pair was equal to the normalized input trace. Each pair of normalized trajectories was then exported to the file format that ebFRET uses to import raw donor and acceptor values. The converted ebFRET compatible traces were then trimmed to exclude portions of the recording before the addition of fluorescent material. The trimmed traces were loaded into the ebFRET Matlab program. Analysis was carried out for 2 to 5 states, with 10 repeats and a precision of 10−20.

After normalization, trajectories from Rad52 addition versus buffer exchange experiments were then trimmed to exclude portions of the recording before the addition of fluorescent material. The trimmed traces were then analyzed separately using ebFRET. The output from ebFRET was then analyzed by KERA v3.0 to sort dwell times of individual events at each state. The dwell times were binned with the first center at 400 ms with a width of each bin of 300 ms. The dwell times were fit to one- and two-phase exponential decays using The GraphPad Prism program. The F-RadTest suggested that single exponential decay was the best fit for dwell-time distributions.

Several modifications were introduced to analyze the data from the surface-tethered RPA. Trajectories were selected for analysis only when (1) there was no signal in the ‘red’ channel, (2) fluorescence terminated in a single-step photobleaching event at least 30 s before the end of the photobleaching period (‘blue’ channel), (3) the signal-to-noise ratio was > 4. Selected trajectories were normalized using emulateFRET with the baseline selected as the portion occurring after photobleaching (final 30 s of the video). After normalization, the trajectories were trimmed at 10 s after photobleaching. Normalized trajectories were analyzed using ebFRET as previously described for the surface-tethered ssDNA.
Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
The data supporting the conclusions of this study are presented in Supplementary Datasets 1–7. Source data for Figs. 1–4, 6, 7 and 9 are available online. Additional data, plasmids for protein expression and code for single-molecule data analysis are available from the corresponding authors upon request.

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| ☑   | State explicitly what error bars represent (e.g. SD, SE, CI) |

Software and code

Policy information about availability of computer code

Data collection
	single-molecule movies were acquired using Single 0.4.

Stopped flow data were analyzed using Kaleidagraph and Kintek Explorer software

Data analysis

IDL 6.2 Student Edition [Commercial software environment used to run scripts [ana_all]], ana_all [Open Source IDL compatible script for extracting trajectories from single molecule movies], MATLAB R2015a - Commercial software environment used to run the following scripts:

- TraceViewer [Custom MATLAB script used to view trajectories extracted from single molecule movies]
- TrimFile [Custom MATLAB script used to trim trajectories in bulk]
- changeToeFRET [Custom MATLAB script used for the normalization of single molecule trajectories in order to make them compatible with eFRET]
- eFRET 3.1.1 [Open Source software for the global analysis of single molecule trajectories using Hidden Markov models]
- KERA 3.0 [Custom script used to extract information, such as dwell times, from eFRET analysis]

Stopped flow data (raw data provided in the source files) were fit using Kintek Explorer or Kaleidagraph with equations described in the Methods section. The parameters used for fitting are outlined in the source files.

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
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- A list of figures that have associated raw data
- A description of any restrictions on data availability

Raw data will be shared upon request. Figures 1-3, Supplemental figures S1-S7 and supplemental dataset figures SD2-SD6 have associated raw data; the processed source data are available with the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

In the single-molecule experiments, the sample size = number of analyzable trajectories in one experiment; in several control experiments we split the trajectories from one experiment in three equal groups. The number of trajectories in each group roughly corresponded in size to the number of trajectories collected in other independent experiments. The number of trajectories and the number of individual dwell times for each experiment are listed in Supplementary tables S1-S4.

For the Stopped flow experiment, each experiment (for example the trace showed in Figure 1 e and f) is an average of 7-8 shots where the protein was mixed with DNA. Such experiments were repeated with three (or more) independent biological samples using separately purified and fluorescently labeled RPA proteins.

Data exclusions

The rules for selecting single-molecule trajectories are explained in the materials and methods section. No selected trajectories were excluded from the analysis.

Replication

Single-molecule experiments were carried out in triplicates.

All stopped flow experiments were repeated with three (or more) independent biological samples using separately purified and fluorescently labeled RPA proteins.

Randomization

All data from each independent experiment that was triplicated were analyzed as a group without randomization. In a few controls, we split the trajectories from one experiment into three groups. In this case, the trajectories were assigned sequentially in each group in order of extraction.

Blinding

When trajectories were assigned to the groups, this was done prior to the analysis and strictly based on the order of extraction.

Reporting for specific materials, systems and methods

Materials & experimental systems

| n/a | Involved in the study |
|-----|----------------------|
| ☑   | Unique biological materials |
| ☑   | Antibodies |
| ☑   | Eukaryotic cell lines |
| ☑   | Palaeontology |
| ☑   | Animals and other organisms |
| ☑   | Human research participants |

Methods

| n/a | Involved in the study |
|-----|----------------------|
| ☑   | ChIP-seq |
| ☑   | Flow cytometry |
| ☑   | MRI-based neuroimaging |