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1 **Single-molecule visualisation of DNA G-quadruplex formation in live cells.**

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18
19
20 **Abstract**

21 G-rich sequences can form alternative DNA secondary structures called G-quadruplexes (G4s).
22 Substantial evidence now exists to support that formation of G4 structures is related to gene-
23 expression and the case for targeting G4s for therapeutic intervention is getting stronger.
24 Nevertheless, there is a need to devise additional approaches to study G4s in living cells to build
25 further understanding on their actual biological relevance. The *in-situ* observation of G4-
26 formation in living cells would provide evidence that goes beyond observations by
27 immunostaining and ChIP-Seq. Herein, we describe a new G4-specific fluorescent probe (SiR-
28 PyPDS) that has properties that enable single-molecule detection of G4s. We use SiR-PyPDS to
29 achieve real-time detection of individual G4 structures in living cells. Live-cell single-molecule
30 fluorescence imaging of G4s is carried out under conditions that use low concentrations of the
31 G4-binding fluorescent probe (20 nM) that enabled us to provide informative measurements
32 representative of the population of G4s in living cells, without globally perturbing G4 formation
33 and dynamics. Single-molecule fluorescence imaging and time-dependent chemical trapping of
34 unfolded G4s in living cells by means of DMS treatment, revealed that G4s fluctuate between
35 folded and unfolded states. We also demonstrated that G4-formation in live cells is cell-cycle
36 dependent and inhibited by chemical inhibition of transcription and replication. The observation
37 of single fluorescent probes binding to individual G4s provides a new experimental perspective
38 on G4-formation and dynamics in living cells. Our imaging approach will help elucidate the
39 putative roles of individual G4s in real-time in the context of living cells.

40

41

42 **Introduction**

43 G-quadruplexes (G4s) are non-canonical structures that can form within guanine-rich nucleic acid
44 sequences (Fig. 1A)^{1,2}. Sequencing of G4s in human genomic DNA (G4-Seq) revealed over
45 700,000 distinct sites that can form G4s, with notable G4-enrichment within gene promoters and
46 at *loci* commonly amplified in cancers³. G4 structures have also been imaged *ex vivo* by
47 immunofluorescence with G4-selective antibodies, both in fixed ciliates⁴ and, more recently, in
48 fixed human cells⁵. The G4-selective antibody BG4 has been used in chromatin immuno-
49 precipitation followed by sequencing (ChIP-Seq), showing that just ~1% of the G4s identified in
50 purified DNA by G4-Seq could be detected within chromatin⁶. ChIP-Seq experiments rely on
51 measurements integrated over millions of cells and therefore provide only an average view of G4-
52 incidence at a given genomic *loci*. However, G4 homeostasis in cells is likely to be regulated by
53 proteins, such as helicases, so *ex vivo* techniques that provide a snapshot of G4 distribution may
54 hide important dynamic processes that can only be observed by live-cell imaging. Fluorogenic
55 G4-binding probes for the detection of both RNA^{7,8} and DNA^{9,10} G4s in living cells have been
56 reported. Generally, such probes are used at relatively high (μM) concentrations which can result
57 in global induction of G4-structures, perturbation of endogenous G4-folding dynamics and
58 cellular stress/toxicity through binding to G4s globally. Furthermore, some observational
59 approaches require environmentally responsive probes which can pose limits on the quantitative
60 study of specific G4-formation as well the challenge of disentangling genuine G4-binding from
61 environmental effects. We have pursued single-molecule fluorescence imaging of G4s in living
62 cells to detect individual G4s in the nucleus of living cells at low nanomolar concentrations of
63 fluorescent probe. The use of a G4-specific probe (SiR-PyPDS) and a control probe (SiR-
64 iPyPDS), with poor affinity to G4s, together with ligand competition experiments, confirmed G4s
65 specificity. Relatively low probe concentrations (nM) helps avoid global induction of G4s
66 inherent in ensemble fluorescence methods. Specifically, only a small fraction (~4%) of G4s are
67 bound by the probe, without perturbing global folding dynamics. Herein we report, for the first
68 time, detection of individual G4s in the nucleus of living human cells by single-molecule
69 fluorescence microscopy.

70 **Results and Discussion**

71 SiR-PyPDS (Fig. 1B) was prepared by tethering the red fluorophore Silicon-Rhodamine (SiR)¹¹
72 to an analogue of an established G4-ligand, pyridostatin¹² (PyPDS), using linkers of different
73 lengths (Fig. S1 and S2). Upon binding to G4-folded oligonucleotides all SiR-PyPDS analogues
74 (Fig. S2) displayed a modest fluorescence increase (~10 fold), which is insufficient to confidently
75 discriminate bound vs unbound probes in cells, but enabled evaluation of optimal linker length by
76 fluorescence titrations. Binding titrations revealed the six carbon linker of SiR-PyPDS (Fig. 1B)
77 as being optimal for G4-binding selectivity of the PyPDS-scaffold, with good binding towards
78 MYC and KIT-1 and h-TELO G4s with K_d values of 0.63 (\pm 0.08) μ M, 1.0 (\pm 0.1) μ M and 2.0 (\pm
79 0.8) μ M respectively, and no detectable binding to double- or single-stranded DNA (Fig. S3).
80 SiR-PyPDS displayed a quantum yield of 0.05 in solution that increases to 0.2 when the molecule
81 is bound to MYC G4 (see methods). We also designed and synthesized a novel PyPDS isomer
82 (SiR-iPyPDS, Fig. 1B) that could act as a poor-G4 binding control in live cells experiments to
83 support unambiguous identification of G4-binding events of SiR-PyPDS. Our control analogue
84 (SiR-iPyPDS) differs from SiR-PyPDS simply for the position of the amino side-chains on the
85 quinoline ring. We reasoned that the steric clash of the side-chains in SiR-iPyPDS could prevent
86 the molecule from adopting the flat conformation required for G-tetrad recognition. Fluorescence
87 titrations confirmed a more than 10-fold lower G4-binding affinity of SiR-iPyPDS compared to
88 SiR-PyPDS (Fig. S4).

89 Given the promising results from ensemble binding experiments by SiR-PyPDS and the negative
90 control analogue SiR-PyPDS, we decided to evaluate the suitability of these probes for single-
91 molecule detection of G4s *in vitro*. To test this, we investigated the binding of SiR-PyPDS or
92 SiR-iPyPDS to a G4-folded oligonucleotide, MYC, immobilized on a PEG/biotin-coated surface,
93 by single-molecule imaging (Fig. 1C-E). We acquired images with a long exposure time (500 ms)
94 to capture only relatively long-lived interactions. At a much lower ligand concentration than what
95 was used in ensemble experiments (250 pM), we could detect on average 867 long-lived SiR-
96 PyPDS spots (Fig. 1F, Supplementary Movie S1) in each field of view (Fig. 1G), but observed a
97 ten-fold reduction in long-lived binding (66 events, $P = 5 \times 10^{-6}$) for the weaker G4 binder SiR-
98 iPyPDS (Fig. 1I, Supplementary Movie S1). We confirmed that events represented binding of
99 individual probes to MYC by observing single-step photobleaching (Fig. S5). As the MYC
100 sequence was also labelled with Alexa Fluor 488 we could use both FRET (Fig. S6A) and single-
101 molecule FRET (Fig. S6B-D) to visualize direct binding of our probe to MYC. Note that at 250
102 pM, the labelled fraction, θ , of G4s is about 0.05%, according to the Hill-Langmuir equation
103 $\theta = [L]/(K_d + [L])$, where $[L]$ is the concentration of ligand and K_d is the dissociation constant

104 for ligand binding to G4s. To further investigate if the number of SiR-PyPDS binding event
105 correlates with the density of G4 targets immobilized on the surface, we varied the surface
106 coverage by mixing the biotinylated MYC G4 with a biotinylated single-stranded DNA strand
107 that does not form a G4, at different ratios (Fig. S7, see Methods). We observed a linear
108 relationship between the number of SiR-PyPDS binding events detected and the concentration of
109 MYC G4 immobilised on the surface, confirming that the number of binding events is
110 proportional to the number of G4s on the surface (Fig. S7) and that this number can be used as a
111 proxy for G4-density. We have also confirmed that the MYC sequence used is folded into a G4
112 structure as judged by circular dichroism spectroscopy (Fig. S8). The observed number of binding
113 events can therefore be used to assess the concentration of folded G4s on the surface. We next
114 compared the binding of SiR-PyPDS and SiR-iPyPDS to different G4-folding oligonucleotides,
115 including MYC, h-TELO and c-KIT-1. Again, we observed a >20-fold increase in the number of
116 binding events for SiR-PyPDS when compared to the control probe SiR-iPyPDS (Fig. S9). These
117 observations confirm that SiR-PyPDS can be applied to single-molecule imaging of G4s and that
118 the decreased binding affinity of the control analogue SiR-iPyPDS causes a lower number of
119 binding events observed (Fig. S9).

120 To further validate that long-lived binding events observed with SiR-PyPDS could be ascribed as
121 G4-specific, we attempted to compete out SiR-PyPDS binding to MYC G4 with an excess of the
122 structurally unrelated G4-binding ligand PhenDC3¹³. Gratifyingly, binding of SiR-PyPDS to
123 MYC was abrogated when an excess (10 μ M) of the potent G4-ligand PhenDC3 was included as
124 a competitor (16 events, $P = 2 \times 10^{-6}$, Fig. 1F, Supplementary Movie S2). Furthermore, we
125 measured the number of binding events displayed by SiR-PyPDS when the G4-folding sequence
126 of MYC was mutated to prevent G4-formation. SiR-PyPDS binding was negligible (23 events, P
127 $= 2 \times 10^{-6}$) for the immobilized single-stranded DNA control (MYC-mut, Fig. 1F, Supplementary
128 Movie S1), which is in agreement with what was observed for SiR-PyPDS ensemble fluorescence
129 titrations (Fig. S3). Both the biotin-MYC and MYC-mut used in this experiment were also
130 functionalized with an Alexa-488 fluorophore. We used the 488 emission to measure the total
131 fluorescence on each coverslip functionalised with either MYC or MYC-mut to ensure
132 comparable density of oligonucleotides on the surface between the different experiments ($\sigma =$
133 10% variation between coverslips, Fig. S7B). Therefore, differences observed in binding events
134 were minimally affected by variations in G4 surface coverage, confirming the suitability of SiR-
135 PyPDS and SiR-iPyPDS control as probes for the single-molecule detection of G4s.

136 We next sought to determine whether the conditions of relatively low probe concentrations used
137 for single-molecule imaging caused global induction of G4-folding or perturbation of G4-folding
138 dynamics. To investigate this, we used G4-folding oligonucleotides (MYC, h-TELO and c-KIT1)
139 labelled with a Cy5 fluorophore at their 5' end and having an overhang hybridised with a
140 complementary oligonucleotide sequence containing a Cy3 fluorophore at its 3' end. The
141 oligonucleotides form a Cy3-Cy5 FRET system capable of assessing the fraction of folded G4s
142 by measuring FRET efficiency between the two fluorophores¹⁴. When titrated with increasing
143 concentrations of PyPDS, no significant FRET perturbation was observed for PyPDS
144 concentrations below 3 μ M (Fig. S10). Therefore, there is no detectable global induction of G4s
145 when imaging under single molecule conditions (Fig 1J). We studied G4-unfolding dynamics
146 using a FRET system with FAM/TAMRA labelled oligonucleotides that were annealed in 150
147 mM K⁺ to form a stable G4 structure (see Methods). We next added a 10 folds molar excess of
148 DNA sequence complementary to the G4-folding sequence to irreversibly trap the unfolded G4
149 sequence as dsDNA. This allowed us to measure the unfolding kinetics by monitoring a
150 concomitant decrease in the FRET fluorescence signal, as previously described¹⁴. We found that
151 μ M concentrations of SiR-PyPDS are required to slow down the unfolding rate for the tested G4s
152 structures and that low nM concentrations used for single-molecule experiments do not globally
153 affect the unfolding rate of the tested G4-structures (Table S1). Our data demonstrate that single-
154 molecule imaging can be used to address the pervasive problem of current G4-detection strategies
155 that use relatively high concentrations of affinity probes that might globally perturb G4 folding
156 and dynamics.

157 We next applied the fluorescent G4-ligands to single-molecule imaging of G4s in live cells (Fig.
158 2A). First, we investigated the toxicity of SiR-PyPDS and SiR-iPyPDS in U2OS cells over a 24 h
159 treatment at different probe concentrations. Both SiR-PyPDS and SiR-iPyPDS did not elicit any
160 cell death response at nM concentrations, as toxicity could only be observed at concentrations
161 higher than 10 μ M (Fig. S11). Based on this, U2OS cells were treated for 30 min with 20 nM of
162 SiR-PyPDS, which resulted in under-labeling of G4s at a density where individual fluorophores
163 were spatially well separated (Fig. 2B). This allowed us to visualize individual probes (SiR-
164 PyPDS or SiR-iPyPDS) binding to targets in the nucleus (Fig. 2B-C, Supplementary Movie S3)
165 using single-molecule imaging (400 frames taken with 100 ms exposure using highly inclined
166 laminated optical sheet (HILO) microscopy)¹⁵. Single step photobleaching provided evidence of
167 binding events by individual probe molecules in the nucleus (Fig. S12), in spite of the extra-
168 nuclear lysosomal accumulation of SiR-PyPDS (Fig. S13). We first measured the number of

169 binding events whereby a SiR-PyPDS molecule remained stationary within a 300 nm radius for
170 three or more consecutive frames (i.e. 300 ms), detecting an average of 79 binding events per
171 nuclei (Fig. 2D). Similarly to what was observed *in vitro*, treatment of U2OS cells with SiR-
172 iPyPDS (20 nM) revealed an average of only 2 long-lived binding events in the nucleus (Fig. 2D).
173 To confirm that differences in the number of nuclear binding events between SiR-PyPDS and
174 SiR-iPyPDS were not due to different cellular uptake of the two ligands, we used confocal
175 microscopy and demonstrated that upon 10 μ M ligands treatment the total nuclear fluorescence
176 intensity measured was comparable between the SiR-PyPDS and SiR-iPyPDS treatments (Fig.
177 S14). These results are consistent with the *in vitro* observations (Fig. 1F) and corroborate the
178 hypothesis that long-lived SiR-PyPDS binding events could be ascribed to specific G4-binding in
179 cells. To further support this hypothesis, we demonstrated that SiR-PyPDS binding could be
180 abrogated in the presence of 10 μ M of the unlabeled competitor G4-ligands PDS¹² or PhenDC3¹³
181 (Fig. S15, Supplementary Movie S4), which is also consistent with what was observed *in vitro*.

182 We next sought to estimate the fraction of G4-labelled by SiR-PyPDS in living cells as we have
183 done for the *in vitro* studies. To do so, we have assumed that the K_d of SiR-PyPDS remains
184 unchanged in the cellular environment and that the nuclear concentration of the probe is 20 nM.
185 Based on these assumptions and using the relationship $[L]/(K_d + [L])$, the fraction of labelled
186 G4s on a single U2OS cells is around 4%. Using this value for labelled G4 fraction, we have
187 roughly estimated the total number of G4s present in a single cell. As we detect about 10 binding
188 events on average in an image frame within a single focal plane ($\sim 1\mu$ m), there would be around
189 100 binding events in an entire U2OS cell of diameter $\sim 10\mu$ m. Therefore, considering we are
190 labelling around 4% of the total number of targets, we can estimate a total number of G4s in a
191 single cell of ~ 3000 , which is in line with what has been detected in human chromatin (between
192 1,000 and 10,000 G4s) by G4-ChIP-Seq experiments.⁶

193 We then compared the temporal dynamics of the interaction between SiR-PyPDS and G4s *in vitro*
194 and in cells, to investigate if characteristic dwell times of SiR-PyPDS binding to G4s *in vitro*
195 could also be detected in cells. Time-lapse imaging was used to observe long-lived events (Fig.
196 3A, Supplementary Movie S5). *In vitro*, an exposure time of 100 ms and interval of 2 s was used
197 to avoid photobleaching effects (time constant $\tau_b = 923$ s), whereas for cellular experiments a
198 slightly longer interval (3 s, $\tau_b = 104$ s) and also a longer exposure time (500 ms) were needed to
199 limit contributions from unbound ligands¹⁶. The histogram of SiR-PyPDS dwell times could be
200 well fitted ($R^2 > 0.99$) to a single exponential distribution, yielding a photobleaching-corrected
201 binding lifetime of 6.6 ± 0.5 s in cells (Fig. 3B), which is significantly shorter (~ 2.5 folds, $P =$

202 4×10^{-7} , unpaired t-test) than that observed *in vitro* for binding (15.4 ± 0.6 s) to MYC G4. To
203 investigate further this apparent discrepancy, we carried out *in vitro* binding experiments with
204 other G4-forming sequences. These experiments indicated that the dwell times for SiR-PyPDS
205 binding to hTelo and c-KIT1 were respectively 2.5 and 2 times shorter than MYC and were
206 comparable to dwell times observed in living cells. These experiments suggested that the binding
207 dynamics of SiR-PyPDS to synthetic G4-forming oligonucleotides observed *in vitro* can be
208 recapitulated in cells, further supporting that our assay can detect endogenous G4s.

209 To gain insight into G4-folding dynamics in living cells, we employed the DNA-methylating
210 agent dimethyl-sulfate (DMS) to irreversibly trap the unfolded G4 state (Fig. 3C). The
211 nucleophilic N7 atoms of guanines are exposed and can be methylated by DMS in single- and
212 double-stranded DNA, but are protected in folded G4s by their participation in Hoogsteen
213 hydrogen bonding (Fig 1A). Thus, transiently unfolded G4s can be methylated and irreversibly
214 prevent further G4 re-folding by blocking Hoogsteen hydrogen bonding at N7s (Fig. 3C). First,
215 we demonstrated that DMS could trap the unfolded G4 state *in vitro* by quantifying binding
216 events of SiR-PyPDS with MYC prior to (300 events) and after (40 events, $P = 0.03$) 20 min
217 treatment with 640 mM (8%) DMS (Fig. 3D). We then examined if a similar DMS-dependent G4
218 depletion could be recapitulated in living cells, while keeping the DMS concentration lower (20
219 mM, 0.25%) to prevent cell death. We observed a time-dependent decrease of SiR-PyPDS
220 binding events in U2OS cells within minutes after DMS treatment (Fig 3E, Supplementary Movie
221 S6), with a ~20-fold reduction ($P = 0.006$) in binding events after 20 min exposure. These results
222 suggest that G4s naturally undergo structural fluctuations in cells. This, in turn, makes their
223 specific detection by chemical methods, such as DMS-Seq¹⁷, ineffective, as they will inevitably
224 trap the unfolded state (Fig. 3C).

225 We further probed dynamic formation of G4s in live U2OS cells through different phases of the
226 cell cycle to gain insights into changes in G4-prevalence during active DNA processing states,
227 such as replication (S phase) and transcription (G1 phase). We first confirmed using confocal
228 microscopy that under different conditions tested there were negligible differences in uptake of
229 the fluorescent G4 ligand (Fig. S14). This ensured that the lack of binding events observed under
230 certain cycle phases or after DMS treatment could be confidently ascribed to a change in G4
231 prevalence. SiR-PyPDS-treated U2OS cells were synchronized to S, G1/S and G0/G1 phases
232 using previously reported procedures⁴ and imaged using single-molecule fluorescence
233 microscopy. During S phase, where the cell is undergoing active replication, significant ($P < 10^{-6}$)
234 binding events could be detected (208 events, Fig. 4A, Supplementary Movie S7). The number of

235 binding events was slightly reduced (103 events, $P = 0.01$) when cells are preparing to initiate
236 replication (G1/S phase) and transcription is active (Fig. 4B, Supplementary Movie S7). There
237 were negligible ($P < 10^{-6}$) binding events during G0/G1 phase (3 events, Fig. 4C, Supplementary
238 Movie S7), where cellular processes are quiescent. These results show that G4 formation is
239 associated with both transcription and replication and is in agreement with previous observations
240 reported in fixed cells^{4,18}. To further confirm the suppression of G4s in the absence of actively
241 processed DNA, we treated unsynchronized U2OS cells with a global replication inhibitor
242 aphidicolin and also the global transcription inhibitor DRB as previously described¹⁷, in order to
243 mimic the quiescent state that characterizes cells undergoing G0 phase. Upon transcription and
244 replication arrest few binding events were detected (3 events, $P < 10^{-6}$), further demonstrating that
245 actively processed DNA is pivotal for G4 formation in living cells (Fig. 4C-D, Supplementary
246 Movie S7).

247 **Conclusions**

248 We have used fluorescent probe molecules to visualise individual G4 structures in living cells, for
249 the first time, using single-molecule fluorescence imaging. The sensitivity of single-molecule
250 methods enabled us to image single binding events to G4 structures at probe concentrations
251 orders of magnitude lower than normally used in biophysical and cellular experiments, thereby
252 minimizing global perturbation of G4s. We applied our new imaging platform to demonstrate that
253 G4-formation is cell-cycle dependent and that the presence of G4s is directly related to
254 fundamental biological processes such as active transcription and replication, as chemical
255 inhibition of these processes led to abrogation of detectable G4s in living cells. Trapping of
256 unfolded G4s by means of DMS methylation revealed that G4s undergo dynamic fluctuations in
257 live cells and that essentially all G4s are trapped in the unfolded state during the course of 20 min
258 DMS treatment (0.25%, 20 mM). We anticipate that further application of this imaging platform
259 will help unravel specific biological functions regulated by individual G4s within the human
260 genome in real-time.

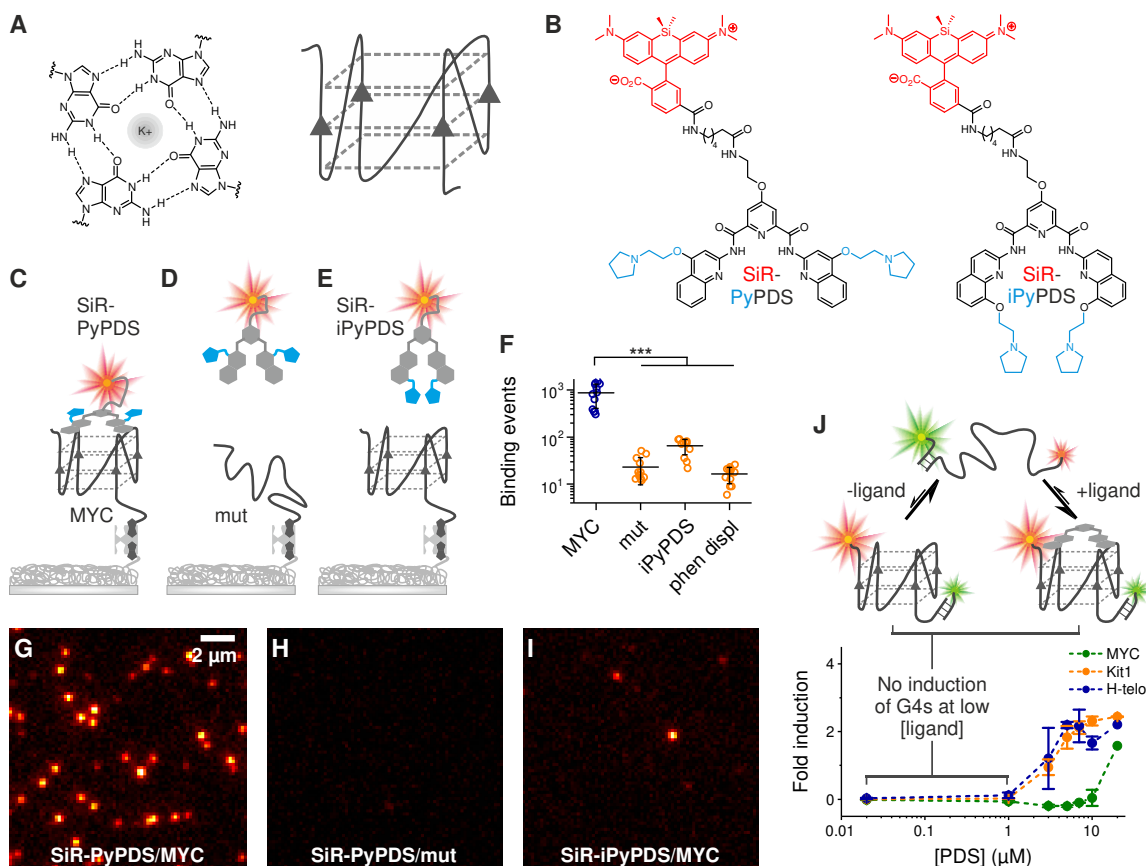
261 **Supplementary Information** is available in the online version of the paper.

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267 **Author Contributions** M.D.A. performed the design, synthesis and biophysical characterization
268 of G4 ligands. A.P. developed the optical setups used for imaging. M.D.A., A.P. and R.T.R.
269 performed *in vitro* imaging experiments. R.T.R. carried out surface preparation for *in vitro*
270 experiments. M.D.A. and A.P. performed imaging experiments in cells. A.P. analyzed imaging
271 data. M.C. A.R. and X.Z. contributed to the synthesis and biophysical validation of the ligands.
272 M.D.A., A.P., R.T.R., S.F.L., D.K. and S.B. contributed to the study design. J.S. assisted with
273 DMS experiments. A.R. contributed to cellular staining and imaging. A.R and L.N characterized
274 the fluorescence properties of the G4 ligands. M.D.A., A.P., D.K. and S.B. interpreted the results
275 and co-wrote the manuscript with input from all authors.

276 **Author information:** The data reported in this paper is available in the main text or in the
277 supporting information. Reprints and permissions information is available at
278 www.nature.com/reprints. S.B. is a founder and shareholder of Cambridge Epigenetix Ltd.
279 Correspondence and requests for materials should be addressed to S.B. (sb10031@cam.ac.uk)
280 and D.K. (dk10012@cam.ac.uk).

281

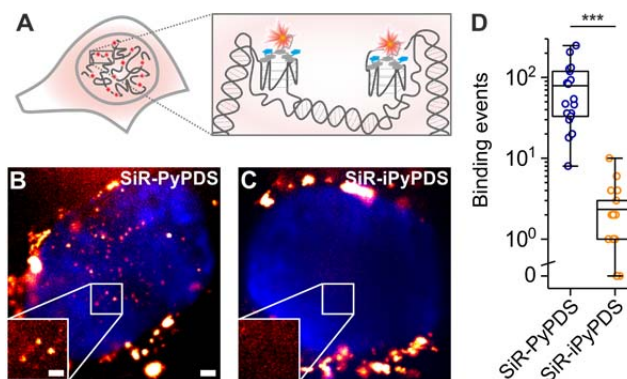


282

283 **Figure 1. *In vitro* single-molecule fluorescence imaging of G-quadruplexes.** (A) Schematic
 284 representation of a G-tetrad (left) and a G4 structure (right). (B) Chemical structure of the
 285 selective G4-fluorogenic ligand SiR-PyPDS (left) and its inactive isomer, SiR-iPyPDS (right).
 286 (C) Schematic of methodology used for visualizing individual G4s. Pre-folded G4 MYC is
 287 attached to a coverslip via a biotin-neutravidin linker. The fluorescent G4-probe SiR-PyPDS
 288 binds to G4 MYC, which can be visualized using single-molecule fluorescence imaging. (D) SiR-
 289 PyPDS will not bind single stranded mutated-MYC that cannot form a G4. (E) The inactive
 290 isomer SiR-iPyPDS with its 10 times reduced binding affinity is less likely to bind G4 MYC. (F)
 291 Quantification of SiR-PyPDS binding to the G4 MYC ii) SiR-PyPDS binding to the mutated-
 292 MYC; iii) SiR-iPyPDS binding to the G4 MYC; iv) SiR-PyPDS binding to the G4 MYC in the
 293 presence of 10 μM unlabeled PhenDC3 competitor. Error bars indicate mean \pm sd. *** $P < 10^{-5}$,
 294 unpaired t-test. (G) Representative images (500 ms exposure) of individual SiR-PyPDS
 295 molecules (250 pM) binding to a surface coated with pre-folded MYC G4 oligonucleotide;
 296 individual fluorescent puncta indicate binding of single SiR-PyPDS molecules. (H) SiR-PyPDS
 297 (250 pM) binding to mutated-MYC. (I) SiR-iPyPDS (250 pM) binding to pre-folded MYC. (J)
 298 Interactions of G4 ligands and G4s can alter the equilibrium between unfolded and folded G4s.

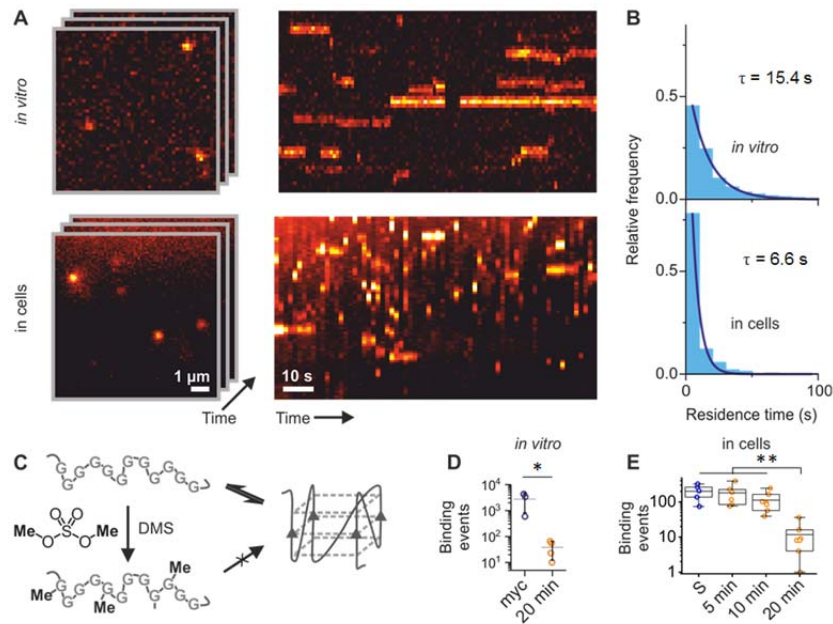
299 Changes in the FRET ratio can be observed at μM PDS concentrations for Kit1 and hTelo and
300 larger concentrations for MYC, indicative of G4 induction, which does not occur at lower
301 concentrations.

302



303

304 **Figure 2. Single-molecule fluorescence imaging of G-quadruplexes in living cells using the**
305 **fluorescent probe SiR-PyPDS.** (A) Schematic of G4s in the cell nucleus with a zoom-in
306 showing G4s stained by SiR-PyPDS. (B) Representative background-subtracted image (max
307 projection of 100 frames with 200 ms exposure) of SiR-PyPDS binding events in a living U2OS
308 cell treated with 20 nM SiR-PyPDS for 30 min before imaging; fluorescent puncta indicate
309 binding of single SiR-PyPDS molecules. Blue color corresponds to nuclear staining with Hoechst
310 33342. Scale bar is 2 μm . Inset scale bar is 1 μm . (C) Representative image of SiR-iPyPDS
311 staining in living U2OS cell treated with 20 nM SiR-PyPDS for 30 min before imaging. (D)
312 Quantification of the binding events within the nucleus lasting more than one frame (100 ms per
313 frame) per cell for SiR-PyPDS and SiR-iPyPDS. Center lines indicate the median; boxes show
314 interquartile range; whiskers denote 5th and 95th percentiles. *** $P < 10^{-5}$, Mann-Whitney U-test.



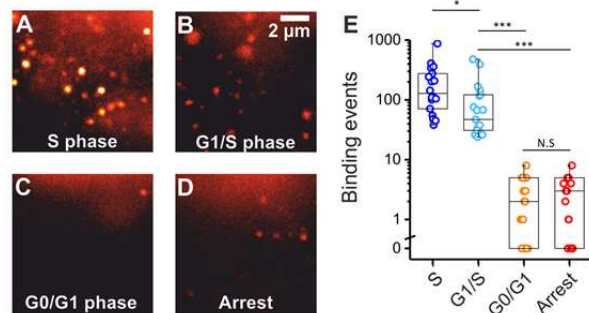
315

316 **Figure 3. G-quadruplexes in living cells undergo dynamic folding/unfolding.** (A) Single-
 317 molecule time-lapse imaging of SiR-PyPDS *in vitro* (top) and in cells (bottom). Individual images
 318 from the time-lapse stack are shown on the left and kymographs on the right show the dynamic
 319 binding kinetics of SiR-PyPDS to G4s. (B) The histograms of dwell times for each experiment (3
 320 positions on a cover slip for *in vitro* and 6 cells for the cell experiment) were fitted with a single-
 321 exponential fit to determine the binding lifetime in each condition. (C) Schematic of DMS-
 322 mediated chemical trapping of unfolded G4s. (D) Quantification of G4-binding events for
 323 untreated and 600 mM DMS-treated G4 MYC for 20 min. Error bars indicate mean \pm sd. * $P <$
 324 0.05, Mann-Whitney U-test. (E) Quantification of G4-binding events detected in living cells upon
 325 increased exposure to DMS (20 mM), showing a clear time-dependent depletion of G4s. Center
 326 lines indicate the median; boxes show interquartile range; whiskers denote 5th and 95th
 327 percentiles. ** $P <$ 0.01, Mann-Whitney U-test.

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333 **Figure 4. The observation of G4s in live cells is altered by the cell cycle phase and**
 334 **transcription.** Representative single-molecule images of G4-binding events are shown for
 335 synchronized U2OS cells in (A) the S phase, (B) the G1/S phase, (C) the G0/G1 phase and (D)
 336 for unsynchronized cells treated with both the transcriptional inhibitor DRB and the replication
 337 inhibitor Aphidicolin. (E) Quantification of binding events lasting more than two frames (100 ms
 338 per frame) per cell in living U2OS cells at different cell-cycle phases and after
 339 transcription/replication arrest. Center lines indicate the median; boxes show interquartile range;
 340 whiskers denote 5th and 95th percentiles. *** $P < 10^{-5}$, * $P < 0.05$, N.S $P > 0.95$ Mann-Whitney U-
 341 test.

342

343

344 **Methods**

345 Detailed synthetic protocols and purification methodologies for the preparation of SiR-PyPDS
 346 and SiR-iPyPDS, biophysical methods and more detailed protocols are described in supporting
 347 information.

348

349 ***In vitro* single-molecule fluorescence imaging**

350 Binding of G4 ligands to synthetic biotinylated oligonucleotides was imaged at single-molecule
 351 resolution by total internal reflection fluorescence microscopy (TIRFM) on glass coverslips
 352 coated with polyethylene glycol (PEG) and NeutrAvidin. In this study, we used two different
 353 PEG coating procedures (see SI): one based on passive adsorption (Coating method 1, used for
 354 data in Fig. 1, 3 and S5) and the other on covalent coupling (Coating method 2, used for data in
 355 Fig. S6, S7 and S9). We found similar surface densities of immobilized biotinylated
 356 oligonucleotides and degrees of non-specific binding on each surface. Buffers for surface
 357 treatment and imaging were freshly filtered each day (0.02 μm syringe filter, Whatman, Cat. No.

358 6809–2101). Each biotinylated surface was then treated in the same way prior to single-molecule
359 imaging. Wells were first coated with 10 μ L of 0.2 mg/mL NeutrAvidin (ThermoFisher, Cat. No.
360 31000) in 1x PBS containing 0.05% tween-20 for 5 min, washed twice with 10 μ L of 1x PBS
361 containing 0.05% tween-20, then treated with 10 μ L of 1x PBS containing 1% tween-20 for 10
362 min. Biotinylated oligonucleotides (c-MYC or c-MYC-mutant, annealed overnight at 100 nM
363 concentration in 100 mM KCl and 50 mM KH₂PO₄, pH 7.4) were then diluted to 10 nM in 1x
364 PBS containing 0.05% tween-20 and 10 μ L added to each well for 5 min. The wells were then
365 washed twice with 10 μ L of 1x PBS containing 0.05% tween-20, then treated with 10 μ L of 1x
366 PBS containing 1% tween-20 for 10 min. The wells were then washed once with 250 pM of G4
367 ligand solutions (SiR-PyPDS or SiR-iPyPDS) in PBS and the solution was finally replaced with 9
368 μ L of G4 ligand at 250 pM in PBS. For *in vitro* ligand displacement experiments, 1 μ L of 1mM
369 PhenDC3 was added to the well. For DMS trapping the pre-annealed MYC oligonucleotide (100
370 nM) was treated with DMS 8% for 20 minutes, quenched by adding 10% β -mercapto-ethanol and
371 used for surface coating.

372

373 The general setup used for TIRFM has been described previously¹⁹. For the *in vitro* experiments
374 TIRFM was implemented on a Nikon Eclipse Ti2 inverted microscope with a Perfect Focus
375 System for maintaining focus during acquisition. 488 nm (MLD 488-200, Cobolt) and 640 nm
376 (LBX-638-180-CSB-PP, Oxxius) lasers were used for excitation with clean-up filters. The
377 emission collected by the 1.49 NA oil immersion 60 \times (90 \times with internal magnification) objective
378 lens (Nikon) was filtered with long-pass and band-pass filters (520/36 – 67030 and 692/40 –
379 67038, Edmund Optics) and imaged on an Evolve 512 Delta EMCCD (Photometrics) with a pixel
380 size of 178 nm, confirmed using a Ronchi ruling. The excitation power density was measured by
381 determining the excitation power after the objective and the beam size in the imaging plane,
382 taking \sim 4-fold near-field enhancement into account. For binding event measurements, a field of
383 view was acquired for each condition with 500 ms exposure time at a power density of 1.4
384 kW/cm². For longer residency time measurements, time lapses of 300 frames were acquired every
385 2 s with an exposure time of 100 ms and a power density of 0.4 kW/cm². For shorter residency
386 time measurements, time lapses of 300 frames were acquired every 100 ms with an exposure time
387 of 100 ms and a power density of 0.4 kW/cm².

388

389 **Live Cell Imaging**

390 In a typical experiment \sim 200,000 U2OS cells diluted in 2 ml of DMEM were plated in a 35 mm
391 dish with a 14mm Glass coverslip at the bottom (MatTek) and allowed to adhere overnight. After

392 ~18h, the media was replaced with 2 ml of fresh DMEM media containing SiR-PyPDS or SiR-
393 iPyPDS at a final concentration of 20 nM and cells were further incubated for 30 min. The
394 DMEM media containing SiR molecules was then discarded and cells were washed 2X with PBS
395 pre-warmed at 37 °C. Finally, the media was replaced with PBS containing Hoechst 2µM for
396 nuclear staining, pre-warmed at 37 °C, which was immediately followed by imaging.

397 The effect of DMS on cellular G4 prevalence was evaluated by treatment prior to SiR-PyPDS
398 labelling: cells were incubated with DMEM containing 20 mM DMS for the indicated time (5, 10
399 or 20 min). After the desired treatment time DMS was quenched by adding 10% β-mercapto-
400 ethanol in PBS followed by 2X washing with PBS pre-warmed at 37 °C.

401 Cell cycle synchronisation was performed with mimosine treatment as previously described⁴.
402 Transcriptional and replication arrest was achieved by co-treatment of cells with DRB and
403 Aphidicolin as previously described¹⁷.

404

405 Binding of SiR-PyPDS to nuclear G4s was visualised using highly inclined laminated optical
406 sheet (HILO) microscopy¹⁵. The microscope setup used has been described previously²⁰. The
407 central plane of the nucleus in U2OS cells was found with either bright-field microscopy or using
408 Hoechst staining. For binding event measurements, 400 frames were acquired for each cell with
409 100 ms exposure time at a power density of 180 W/cm². For residency time measurements, time
410 lapses of 70 frames were acquired every 3 s with an exposure time of 500 ms and a power density
411 of 180 W/cm².

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