Cellular visualization of G-quadruplex RNA via fluorescence lifetime imaging microscopy

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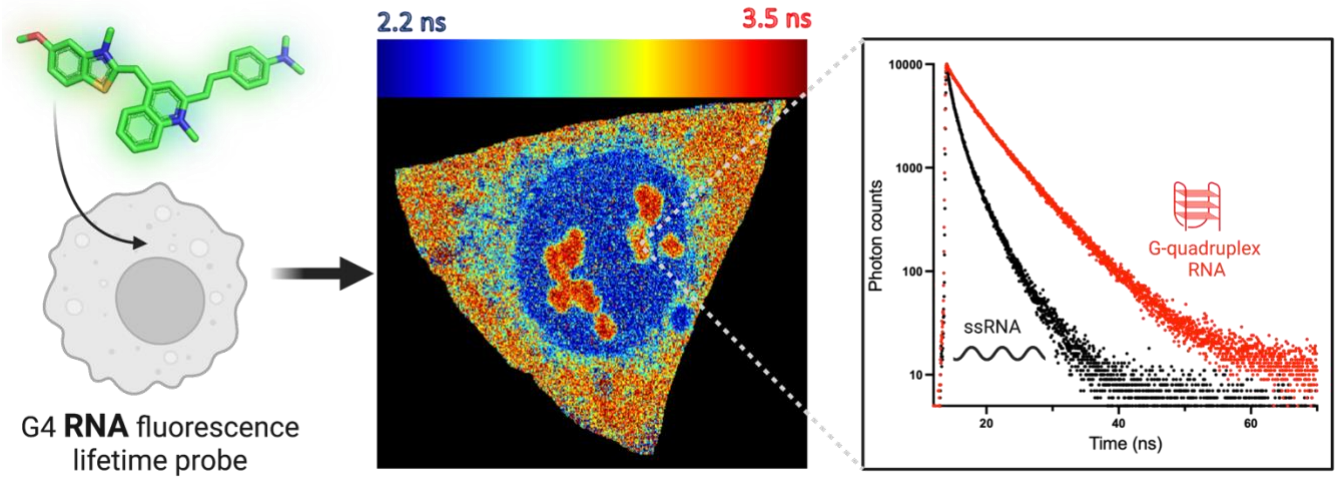
ABSTRACT: Over the last decade, appreciation of the potential roles of G-quadruplex (G4) structures in cellular regulation and maintenance have rapidly grown, making the establishment of robust methods to visualize G4s increasingly important. Fluorescent probes are commonly used for G4 detection *in vitro*, however, achieving sufficient selectivity to detect G4s with confidence in a dense and structurally diverse cellular environment is challenging. The use of fluorescence probes for G4 detection is further complicated by variations of probe uptake into cells, which may affect fluorescence intensity independently of G4 abundance. In this work, we report an alternative small-molecule approach to visualize G-quadruplexes that does not rely on fluorescence intensity switch-on and thus, does not require the use of molecules with exclusive G4 binding selectivity. Specifically, we have developed a novel thiazole orange derivative, **TOR-G4**, that exhibits a unique fluorescence lifetime when bound to G4s compared to other structures, allowing G4 binding to be sensitively distinguished from non-G4 binding, independently of local probe concentration. Furthermore, **TOR-G4** primarily co-localizes with RNA in the cytoplasm and nucleoli of cells, making it the first fluorescence lifetime-based probe validated for exploring the emerging roles of RNA G-quadruplexes *in cellulo*.



INTRODUCTION

The structural landscape of DNA and RNA is essential in facilitating the cellular function of nucleic acids, above and beyond their key role in carrying genetic information, making nucleic acid secondary structures critical mediators in the regulation of essential cellular processes.[1–3] G-quadruplexes (G4s) are secondary structures that form in DNA and RNA through non-canonical hydrogen bonding between four guanine bases.[4,5] As understanding of pathways regulated by nucleic acids has evolved, G4s have progressed from being considered as elusive structures that form in repeat sections of telomeres,[6] to prospective regulators of cell biology that form extensively across cells.[4,5]

In the nucleus, DNA G4s have been associated with epigenetic regulation of gene expression, through their interactions with regulatory proteins, such as transcription factor and chromatin modifiers.[7,8] Whilst RNA G4s have been linked to regulation of RNA splicing, transport and translation, as well as RNA-mediated stress responses in the cytoplasm.[9–11] As many of these processes occur in spatially confined regions of the cell,[9–11] deciphering the biological roles of G4s now requires visualization not just within individual cells, but within restricted subcellular compartments. It is thus necessary that the tools we use to interrogate G4s formation continue to evolve as our study of G4 biology becomes simultaneously more wide-ranging and precise.

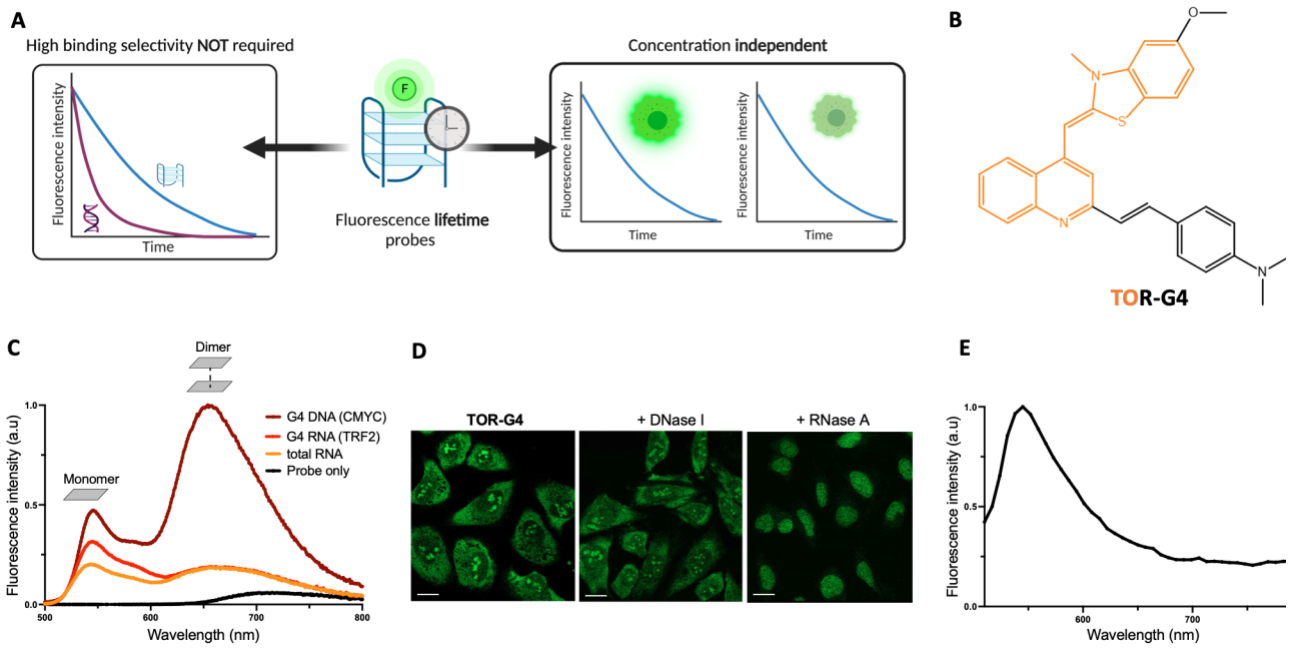


Figure 1 – A) Schematic of the principles and benefits of using fluorescence lifetime-based detection for G4 imaging: binding to each nucleic acid topology is characterized by a unique time-resolved decay and lifetime e.g. longer for G4 (blue), shorter for duplex (purple), therefore each topology can be clearly detected even in the presence of the other, and high binding selectivity is not required. Additionally, the measurement is independent of probe concentration. B) Structure of thiazole orange derivative **TOR-G4** - thiazole orange core is shown in orange. C) *In vitro* emission spectra of **TOR-G4**, free in solution (black) and bound to G4 DNA (maroon), G4 RNA (red) and total RNA (orange), following 470 nm excitation. D) Confocal images of **TOR-G4** in fixed U2OS cells before and after treatment with DNase I or RNase A. The RNase treatment causes significant changes in the probe's distribution in cells. Scale bar = 20 µm. E) Cellular emission spectra of **TOR-G4** in U2OS cells following 514 nm excitation.

The first direct proof of G4 formation within cells was provided using G4-specific antibodies visualized by immunostaining.[12–14] In parallel, several small-molecule 'switch-on' probes have been reported that become fluorescent upon binding to G4s.[15–17] Whilst such fluorescence intensity probes are very useful for *in vitro* studies, their use for understanding G4 biology in cells presents several challenges. Exceptionally high G4

selectivity is required for such probes to work effectively within cells, due to the large abundance of non-G4 secondary structures that may result in a smaller 'switch-on' which is, however, sufficient to produce false positive fluorescent signals.[2,3] Not only is this high G4 binding affinity difficult to achieve, but may in fact alter natural G4 formation within cells.[18] Secondly, fluorescence intensity is intrinsically concentration dependent,[19] and so may vary independently of G4 content due to differences of probe uptake into a given cell or organelle.

To address the limitations of fluorescence intensity probes, alternative methods have arisen to visualize G4s based on changes to the fluorescence lifetime of a molecule upon nucleic acid binding.[20–23] For environmentally sensitive fluorophores, the rate of fluorescence decay can vary depending on the binding conformation and the environment of a molecule.[24] Such variations in fluorescence lifetime make it possible to identify binding to specific structures, even when using less selective molecules that interact promiscuously with multiple topologies (Figure 1A). Additionally, fluorescence lifetime is generally concentration-independent and therefore remains constant regardless of cellular uptake (Figure 1A).[24] Currently, there are a limited number of fluorescence lifetime-based probes that have been reported for visualizing G4s,[20–23] which have enabled understandings of G4 dynamics within cells such as G4 unfolding by the action of DNA helicases.[21] However, previous G4 lifetime probes were developed for DNA G4s, which has left the emerging role of RNA G4s inaccessible for study using lifetime-based approaches.

In this work, we designed **TOR-G4** - a derivative of thiazole orange (Figure 1B), and explored its use as a new G4 fluorescence lifetime probe. Thiazole orange (TO) was selected as the core motif of the probe due to its reported selectivity for G4s.[25–28] Additionally, thiazole orange is known to be an environmentally sensitive probe where rotation around the bridging single bond leads to fast relaxation of the excited state and thus, a short fluorescence lifetime; [29,30] however, on binding to DNA, this rotational freedom is limited resulting in a significant increase in fluorescence intensity and lifetime.[29,30] The lifetime sensitivities of TO thus make it a promising starting point to develop new lifetime probes for G4 imaging.

Here, we demonstrate that the lifetime of **TOR-G4** is highly dependent on the interacting nucleic acid structure – being highest in the presence of G4s and lower for other sequences. Within cells, we showed that the probe primarily co-localizes with RNA and displays fluorescence lifetimes which are dependent on G4 binding, making it the first lifetime probe suitable for detecting RNA G4s within cells. Overall, we present **TOR-G4** as a novel molecule for visualizing G4 RNA that overcomes many of the limitations of intensity-based switch-on probes.

RESULTS AND DISCUSSION

Characterization of a new thiazole orange derivative

Several previous reports have shown that structural modifications of thiazole orange can lead to increased G4 selectivity.[25,27,28] Thus, we designed and synthesized **TOR-G4** (Figures 1B, S1-S5) which includes the addition of a benzyl-styryl unit to extend electron conjugation of the original TO motif and thus, its potential for π-stacking with G-quartets. A methoxy and dimethylamine group were incorporated to increase the potential for non-covalent interactions with G4 loops.

After successful synthesis and characterization of **TOR-G4**, we next investigated the photophysics of the molecule in the absence and presence of various nucleic acid structures (Figures 1C, S6). In aqueous media, the probe displays a very weak emission centred at *ca*. 700 nm, however, the addition of nucleic acids results in a significant increase in the fluorescence intensity with two spectral peaks visible, centred around 540 nm and 660 nm (Figure 1C). As thiazole orange is known to self-assemble into aggregates in aqueous solution,[31–34] we hypothesized that the two emission peaks were due to the monomer and dimer/aggregated form of the probe.

To investigate probe aggregation, we first measured the excitation spectra at both peaks and observed distinct spectra corresponding to separate ground state entities (Figure S7). The species emitting at 660 nm had a blue-shifted excitation maximum relative to the species emitting at 540 nm, which is consistent with H-aggregate emission at 660 nm.[35] To further confirm that the 660 nm peak arises from probe aggregates, we performed a concentration titration where the concentration of **TOR-G4** was increased in the presence of the DNA G4 sequence CMYC (Figure S7). We observed that the intensity of the 660 nm peak increased rapidly with increasing probe concentration: this change in the peak ratios shows that the species emitting at 660 nm is favoured by higher concentrations of **TOR-G4** and is therefore likely to correspond to the aggregated form of the probe. The monomer form of the probe (emitting at 540 nm) only appears after the addition of oligonucleotides, potentially due to stronger interactions of the probe with DNA/RNA than with itself (Figures 1C, S7).

TOR-G4 localizes with RNA within cells

We next set out to characterize the cellular uptake and localization of the molecule. We opted to characterize **TOR-G4** within fixed cells, which are commonly used in the field when imaging G4s via immunofluorescence,[12,13,36] due to the observed toxicity of the molecule (Figure S8, GI_{50} = 143 nM). Confocal microscopy images of the probe within U2OS cells revealed a distinct staining pattern, with particularly high intensity in the cytoplasm and nucleoli, which is indicative of RNA binding (Figure 1D). To confirm this preferential binding to RNA, we performed nuclease experiments where cells were treated with either DNase I or RNase A to remove cellular DNA and RNA, respectively. We found that the treatment with DNase did not substantially alter neither the localization nor the fluorescence intensity of the probe (Figure 1D). In contrast, treatment with RNase resulted in significant changes in the staining pattern of the probe, leaving solely nuclear staining without characteristic nucleolar staining (Figure 1D).

The cellular localization results suggest that **TOR-G4** naturally localizes with RNA within cells and is not significantly affected by the removal of DNA. However, in the absence of RNA, the probe binds to DNA, in turn becoming a nuclear stain. This preferential colocalization of **TOR-G4** with RNA makes the molecule one of a limited number of probes well suited for studying G4 RNA within cells using fluorescence microscopy.[37–40]

Interestingly, the fluorescence spectrum recorded of **TOR-G4** within U2OS cells shows only the presence of the monomer species emitting at 540 nm (Figure 1E). The exclusive existence of the monomer species in cells may be due to the high density of interacting nucleic acids. The high cellular ion concentration may also contribute, which we found promotes probe disaggregation (Figure S9).

We considered that probe disaggregation may also occur upon binding to other biomolecules that are present in cells, such as proteins and lipids. To investigate interactions with other cellular components, we measured the emission spectra of the probe in U2OS cell lysate before and after the treatment with nuclease. We found that in total lysate, two emission peaks at ~550 nm and 650 nm were observed in the spectrum (Figure S10), consistent with the presence of both the monomer and the aggregate species. In contrast, after treatment with

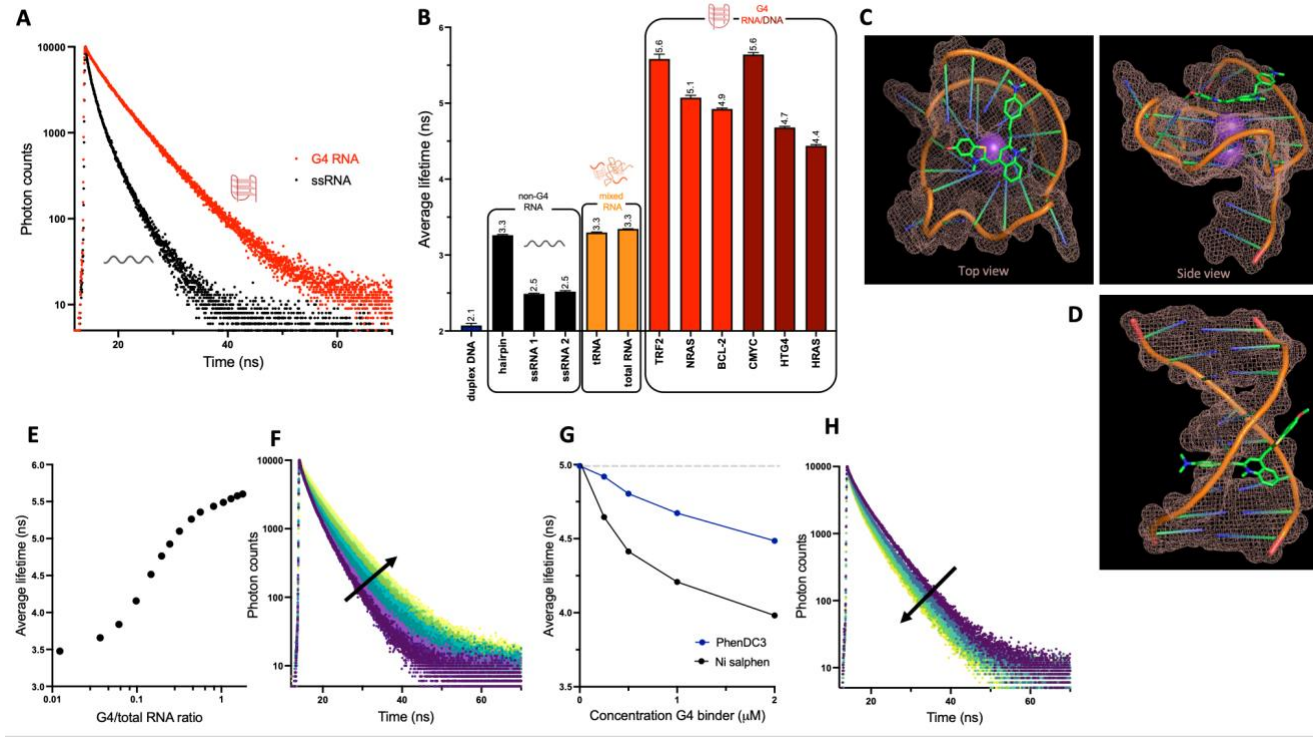


Figure 2 – A) Sample time-resolved fluorescence decays of **TOR-G4** when bound to a G4 or a single-stranded RNA sequence. B) Average fluorescence lifetimes of **TOR-G4** when bound to various nucleic acid structures after excitation at 467 nm and detection at 540±32 nm. C) Molecular modelling of **TOR-G4** bound to the G-quadruplex CMYC or D) a duplex DNA structure demonstrating variations in the probe's binding conformation. E) Average fluorescence lifetimes and F) time-resolved fluorescence decays of **TOR-G4** recorded in the presence of varying ratios of G4 RNA to total RNA. A significant increase in lifetime is detected at a G4 to total RNA ratio >0.1. G) Average fluorescence lifetimes recorded from displacement assays – displacing **TOR-G4** from an RNA G4 sequence with established G4 binders Ni-Salphen and PhenDC3 (see Figure 3 for molecular structures). H) Time-resolved fluorescence decays of **TOR-G4** during G4 displacement assay with Ni-Salphen.

nuclease, only a single peak corresponding to the probe aggregates (emitting at ~650 nm) was observed. This demonstrates that to observe the monomer species, specific binding to nucleic acids is required. The probe is thus well suited for specifically studying nucleic acids within cells without interference from other biomolecules.

Fluorescence lifetime of TOR-G4 varies based on nucleic acid structure

Having confirmed that **TOR-G4** stains RNA in cells and emits from its monomer state upon binding, we set out to test whether it may be used as a fluorescence lifetime probe for G4s. We measured the time-resolved fluorescence traces of the cellularly-relevant monomer species in the presence of multiple structures, including six G4-forming sequences, considering both RNA and DNA structures. As a baseline measurement, we also measured the probe's lifetime in the presence of total RNA extracted from yeast, which contains a mixture of all RNA structures present in a typical cell. Similarly, we tested tRNA cell extract as an additional subset of mixed RNA topologies, including G4s.[41] Next, we selected RNA sequences that cannot form G4s, such as simple single stranded RNA sequences, which lack secondary structure, as well as a hairpin RNA sequence. Finally, we recorded the lifetime of **TOR-G4** bound to duplex DNA extracted from calf thymus to consider lifetime selectivity for RNA over DNA.

We found that the time-resolved fluorescence decays of **TOR-G4** fit well to a bi-exponential decay function (Figures 2A, S11 and S12). Both lifetime components were highly dependent on the nucleic acid structure the probe interacted with, being significantly higher in the presence of G4 structures than for mixed or non-G4 topologies. In the presence of G4 structures, the decay of **TOR-G4** also had higher contributions from the longer lifetime component (amplitude 2 and tau 2, Figure 12), causing the probe to have a significantly higher weighted average lifetime when bound to G4s (4-6 ns) compared to non-G4/mixed topologies (2-3 ns) (Figures 2A, B). The fluorescence enhancement of the probe upon DNA and RNA binding also displayed similar trends to that of the fluorescence lifetime, being the highest when the probe was bound to G4s – although there was significantly higher variance in the intensity-based measurements (Figure S13). Despite preferential cellular binding to RNA, the lifetime values for G4 DNA and RNA were comparable, suggesting that **TOR-G4** has selectively high lifetimes for all G4 structures regardless of the nucleic acid.

To rationalize the structural dependence of **TOR-G4**'s lifetime, we performed molecular modelling studies comparing the DFT-optimised conformation of the probe when bound to the structure that produced the lowest lifetime (duplex DNA) and the highest lifetime (the G-quadruplex CMYC) (Figures 2C, D). Docking studies showed that when interacting with the duplex structure, a portion of the probe intercalates in between base pairs, however, due to the extended size of the molecule, part of the probe is forced to point away from the DNA backbone. This displaced fragment likely has a substantial amount of rotational freedom, which is likely to increase the rate of non-radiative decay[24] and result in a lower probe lifetime. In contrast, when bound to the G4 structure via end-stacking, the entirety of the probe fits on top of the tetrad. Such a binding arrangement means that the whole of the molecule is involved in the π-stacking interaction with the G4 and is thus more conformationally restricted, likely leading to a longer lifetime.

To explore the hypothesis that differences in lifetime are explained by differing binding interactions, we performed binding affinity titrations on selected sequences (Figures S14 and S15). From this analysis, we found the K_a of **TOR-G4** was indeed 2-3× higher for G4 sequences than for other structures. Interestingly, we also noted that the binding affinity for duplex DNA was comparable to that of total RNA – suggesting that the cellular localization with RNA is not due to specific RNA binding preferences. Instead, within cells intercalation into DNA may be impaired due to chromatin structure, as has been previously described for other intercalating dyes,[42–44] causing the probe to interact primarily with RNA which is abundant in the nucleoli and cytoplasm.

Perturbing G4 binding alters lifetime of TOR-G4

Having established that the lifetime of **TOR-G4** varies depending on nucleic acid structure, we next tested if the lifetime of the probe could dynamically respond to changes in G4 binding. We opted for two perturbation strategies to either increase or decrease G4 binding. Firstly, to assess the lifetime response to increasing G4 prevalence, we measured the time-resolved decays of **TOR-G4** in a solution of total RNA and investigated how the addition of an RNA G4 sequence affected the detected lifetime of the molecule. We found that the lifetime gradually increased from 3.5 to 5.5 ns with increasing G4 concentration (Figure 2E, F). Moreover, it took a less than 5% increase in G4 concentration to observe a significant increase in the probe's lifetime, demonstrating the high G4-sensitivity of **TOR-G4**.

Secondly, to investigate how the probe responds to reduced G4 binding, we performed a G4 displacement assay using an RNA G4 sequence. To achieve this, we used two validated G4 ligands that are structurally distinct,

PhenDC3 [45,46] and Ni-Salphen[47] (Figures 3D,E) and titrated them into a solution of **TOR-G4** pre-bound to an RNA G4. As expected, we observed that increasing the concentration of known G4 binders displaced the probe from its RNA G4 substrate, resulting in a significant drop in fluorescence lifetime, by 1 ns with Ni-Salphen and 0.5 ns with PhenDC3 (Figure 2G,H). In contrast, when repeating the same experiment using a hairpin RNA sequence as a **TOR-G4** binding substrate, we did not observe any significant change in the probe lifetime (Figure S16). Additionally, displacement from total RNA resulted in a modest drop in lifetime (by 0.3 and 0.1 ns when adding Ni-Salphen and PhenDC3, respectively, Figure S15), which is in agreement with total RNA containing a mixture of G4 and non-G4 structures. Overall, these experiments demonstrate that the lifetime of **TOR-G4** is highly sensitive to G4 binding and may be used to assess G4-prevalence within cells.

TOR-G4 detects G4 content within cells via FLIM

After the *in vitro* validation of **TOR-G4** as a successful G4 fluorescence-lifetime probe, we next considered its suitability for detecting G4 abundance in a cellular environment via Fluorescence Lifetime Imaging Microscopy (FLIM). Here, the fluorescence lifetime is calculated for individual pixels of a confocal image to yield a colour-coded map of pixel lifetimes, where high lifetime pixels are shown in red and lower lifetime pixels in blue (Figure 3). FLIM analysis of **TOR-G4** in the osteosarcoma cell line U2OS, revealed that the lifetime of the probe varies

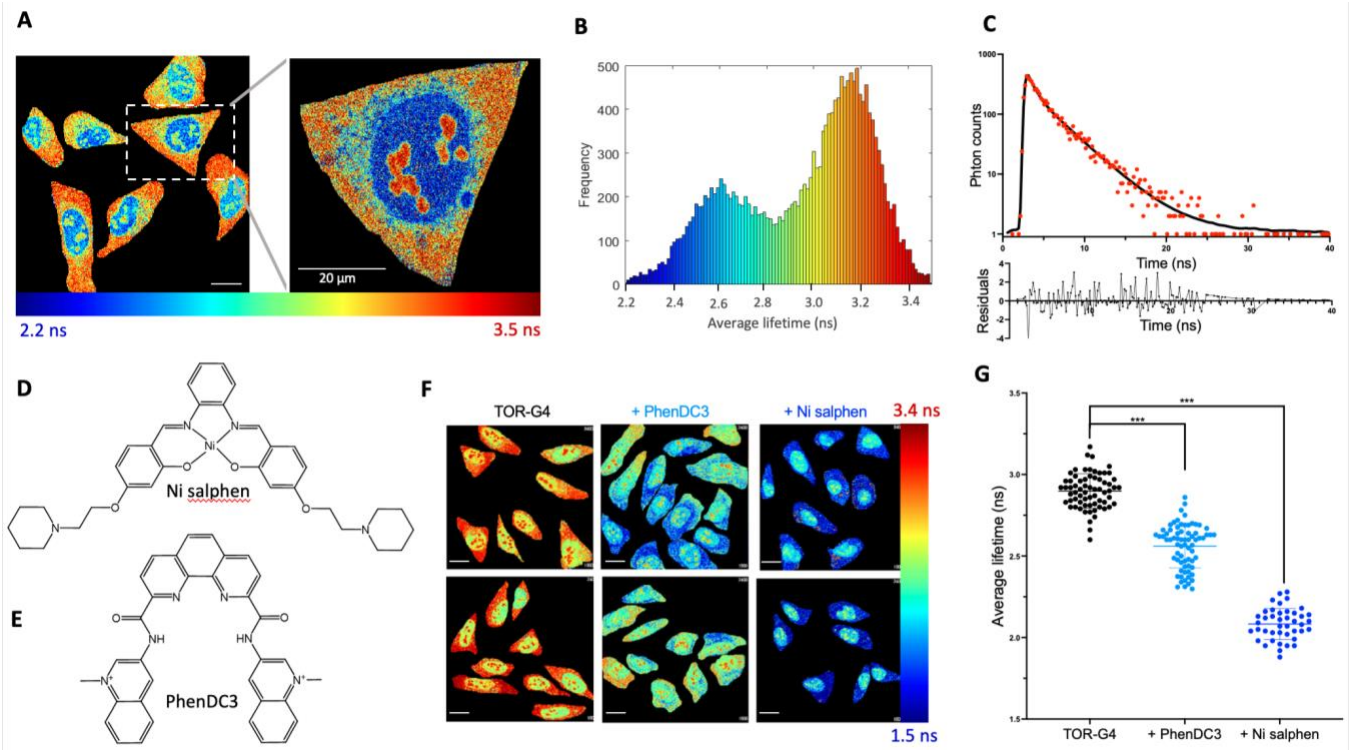


Figure 3 - A) FLIM images of **TOR-G4** in U2OS cells. B) Histogram of pixel frequency against average fluorescence lifetime of **TOR-G4** within U2OS cells. C) Example of cellular fluorescence decay and decay fit residuals of **TOR-G4** to a biexponential decay function. D) Structure of Ni-Salphen and E) PhenDC3. F) FLIM images from two biological repeats of U2OS cells incubated with **TOR-G4** before and after the addition of PhenDC3 or Ni-Salphen. G) Average cellular lifetime from G4 displacement assay with PhenDC3 and Ni-Salphen. Excitation at 477 nm and emission collected across 550-700 nm. Scale bars = 20 μm.

considerably across the cell (Figure 3A-C). Specifically, there were two distinct lifetime environments (Figure 3B): the first at 3.2 ns in the cytoplasm and nucleoli, which is consistent with the *in vitro* measurements of the probe bound to total RNA (i.e. a mixture of G4 and non-G4 binding). In comparison, a significantly lower lifetime

is seen for the non-nucleolar parts of the nucleus where there is a peak lifetime of 2.6 ns. This lower lifetime is comparable to that measured with single stranded RNA *in vitro* and may be due to binding of the probe to mRNA transcripts in the nucleus, or alternatively may represent residual duplex DNA binding

We next sought to validate that the lifetime measured within each whole cell does not vary based on the probe uptake. To assess this, we measured the average fluorescence intensity within each cell - which reflects the internal probe concentration - and correlated it with the average cellular lifetime. Interestingly, we found no correlation between the average intensity of a cell and its average lifetime (Figure S17), demonstrating that the fluorescence lifetime of **TOR-G4** is a robust measurement which is not affected by fluctuations in cellular probe concentration.

Finally, to test the sensitivity of the probe towards G4 binding within cells, we performed cellular G4 displacement assays. Similarly to the *in vitro* assay, two G4 binders PhenDC3 and Ni-Salphen (1 µM, Figure 3D, E) were added to cells which had been pre-incubated with **TOR-G4**. After 4 hours of incubation with the corresponding G4 binders, significant drops in the fluorescence lifetime were observed (Figure 3F, G), with Ni-Salphen resulting in a larger drop in lifetime compared to PhenDC3 – thus showing the same trend as the *in vitro* displacement results. This data demonstrates that perturbations to G4 binding within cells results in significant changes in the lifetime of **TOR-G4**, making the probe an effective and concentration-independent tool for monitoring changes in cellular G4 formation.

DISCUSSION

The formation of G-quadruplex structures in DNA and RNA has been linked to multiple regulatory pathways across the cell, in increasingly diverse and compelling ways.[7–11] G4s are thus promising therapeutic targets for diseases including cancer and neurodegeneration.[48,49] Untangling the role of G4s in cellular processes has been facilitated by G4 visualization using either small-molecule fluorescent probes or fluorescently-labelled antibodies.[12–17,36]

Historically, research on G4s has been focused on DNA G4s which is reflected in the greater availability of fluorescent probes that exhibit nuclear staining.[50] In comparison, visualization of RNA G4s (rG4s) is still a relatively new area of study.[37–40] Previously, it was suggested that RNA G4s may even be globally unfolded within cells due to the persistent activity of G4 helicases.[51] However, more recent studies have provided extensive evidence that rG4s not only exist within cells but may be pivotal for mediating RNA interactions with proteins that control processes such as splicing, translation and stress granule formation. [9–11]

Despite the exciting emerging roles of RNA G4s within cells, imaging rG4s poses a unique challenge due to the increased structural diversity of RNA compared to DNA.[1] This means that any rG4 probes must exhibit exceptionally high binding selectivity for G4s. Additionally, as rG4s are often found in phase-separated regions of the cell (and may even drive phase separation events),[52–54] probe uptake must be considered when interpreting fluorescence images. For example, differences of probe uptake into phase-separated organelles confound measurements of fluorescence intensity, where increased fluorescent signal may be due to local probe concentration rather than G4 abundance.

The use of Fluorescence Lifetime Imaging Microscopy is a strategy that can be used to overcome stringent requirements for G4 binding selectivity as it allows for binding interactions with G4s to be distinguished from that of other structures and, furthermore, provides a readout of G4 abundance which is independent of local probe concentration. Whilst some probes have been reported to display distinct fluorescence lifetimes when bound to G4 DNA compared to other structures,[20–23] lifetime probes for RNA G4s have yet to be reported. In this work, we have filled this gap by describing a fluorescence lifetime-based probe suitable for imaging RNA G4s in cells.

We first characterized the fluorescent properties of **TOR-G4**, noting that *in vitro* the probe can exist in either a monomeric or an aggregated form, whilst in cells the molecule fully disaggregates, presumably upon nucleic acid binding. The fluorescence lifetime of the monomer species is highly dependent on the structure of the interacting nucleic acids, being the highest when bound to G4 structures, compared to non-G4 or mixed topologies. These differences in the probe's lifetime were rationalized with molecular modelling studies showing that the conformational flexibility of **TOR-G4** is restricted when bound to a G4, compared to a duplex structure, which would in turn reduce the rate of non-radiative decay.

Within cells, **TOR-G4** predominantly stains the cytoplasm and nucleoli and is sensitive to treatment with RNase A. Whilst *in vitro*, there was no binding selectivity for RNA over DNA, this colocalization with RNA within cells may be explained by impaired intercalation into chromatinized DNA,[42–44] making the probe most suited for studying RNA G4s. To validate the sensitivity of **TOR-G4** to G4 content within cells, we performed G4 displacement assays with known G4 ligands PhenDC3 and Ni-Salphen. We demonstrated that displacing **TOR-G4** from G4s results in a large and significant drop in the average cellular fluorescence lifetime, confirming that the molecule can be used as a sensitive tool to probe cellular rG4 abundance.

CONCLUSION

In this work we present the characterization and application of **TOR-G4** – a small-molecule suitable for imaging RNA G4s via FLIM. By characterizing the fluorescence lifetime of **TOR-G4** with a range of G4 and non-G4 structures, we established that the probe displays distinct fluorescence lifetimes when bound to G4s compared to other structures, which can be perturbed by the addition of known G4 ligands. In cells, the probe primarily localizes in cellular compartments that are rich in RNA, making it one of a limited number of molecules suitable for probing RNA G4 formation. Overall, **TOR-G4** represents an alternative, concentration-independent tool for visualizing RNA G4s, which overcomes the need to obtain exclusive G4 binding selectivity within cells

ASSOCIATED CONTENT

Supporting Information

Description of experimental protocols, materials and supplementary figures (S1-S17) as mentioned in the text (PDF).

AUTHOR INFORMATION

Author Contributions

J.R., M.K.K. and R.V. designed the study with input from M.D.A. on cellular experiments. J.R., M.D.A., M.K.K., and R.V. co-wrote the paper. J.R., S.G.S and K.M. performed experiments and analysed the data. M.D.A., M.K.K. and R.V. secured the funding for this work.

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ABBREVIATIONS

G4	G-quadruplex
rG4	RNA G-quadruplex
K_a	Association constant
FLIM	Fluorescence Lifetime Imaging Microscopy

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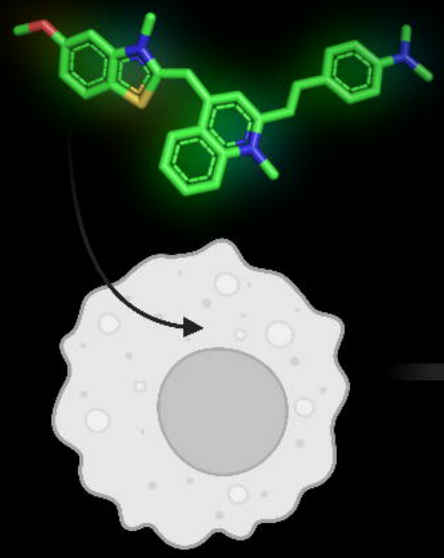
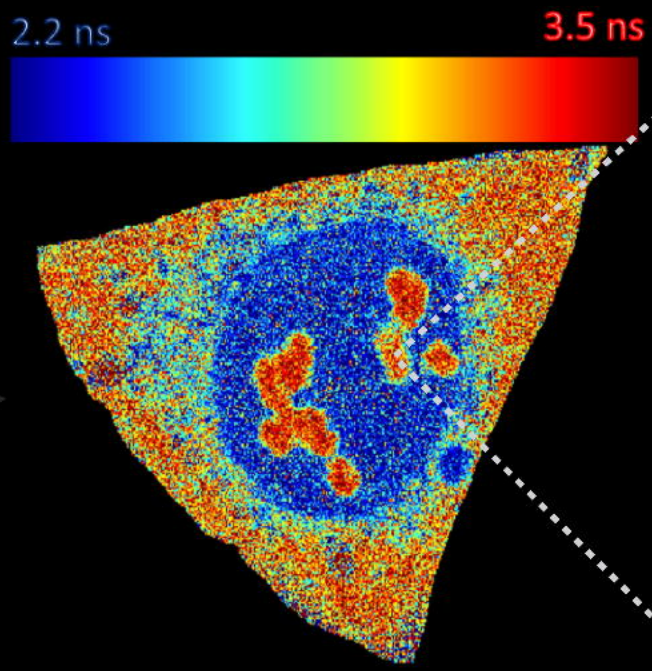
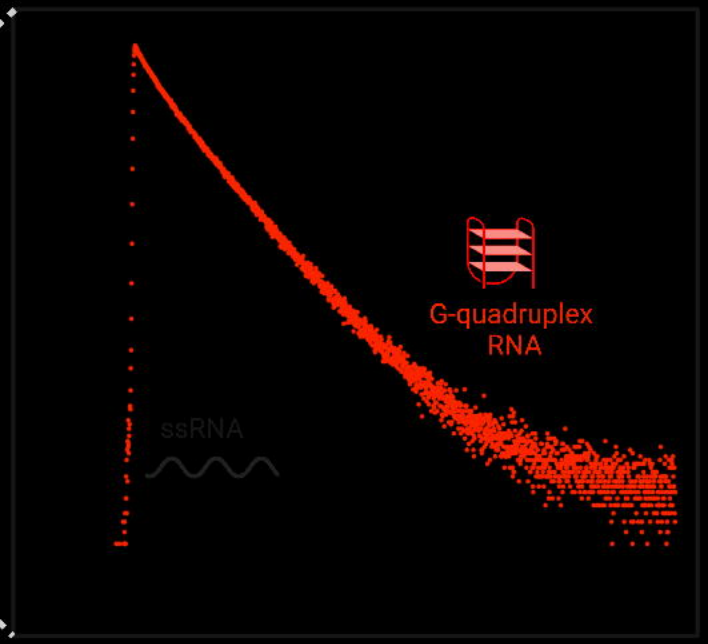
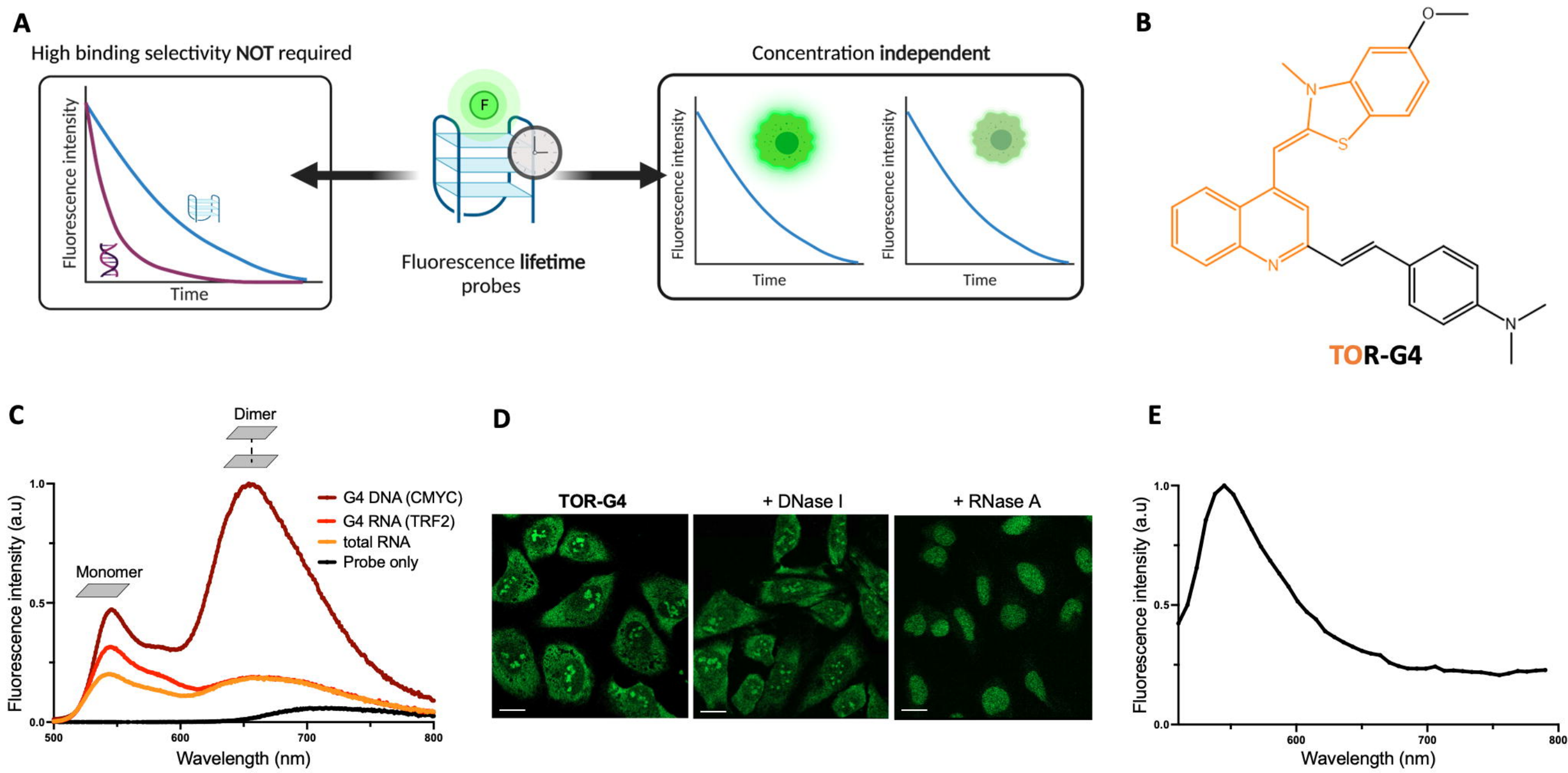


G4 **RNA** fluorescence lifetime probe






A
High binding selectivity NOT required
Fluorescence intensity
Time
F
Fluorescence lifetime probes
Concentration independent
Fluorescence intensity
Time
Fluorescence intensity
Time
B
O
N
S
N
N
TOR-G4
C
Dimer
Monomer
G4 DNA (CMYC)
G4 RNA (TRF2)
total RNA
Probe only
Fluorescence intensity (a.u)
1.0
0.5
0.0
500
600
700
800
Wavelength (nm)
D
TOR-G4
+ DNase I
+ RNase A
E
Fluorescence intensity (a.u)
1.0
0.5
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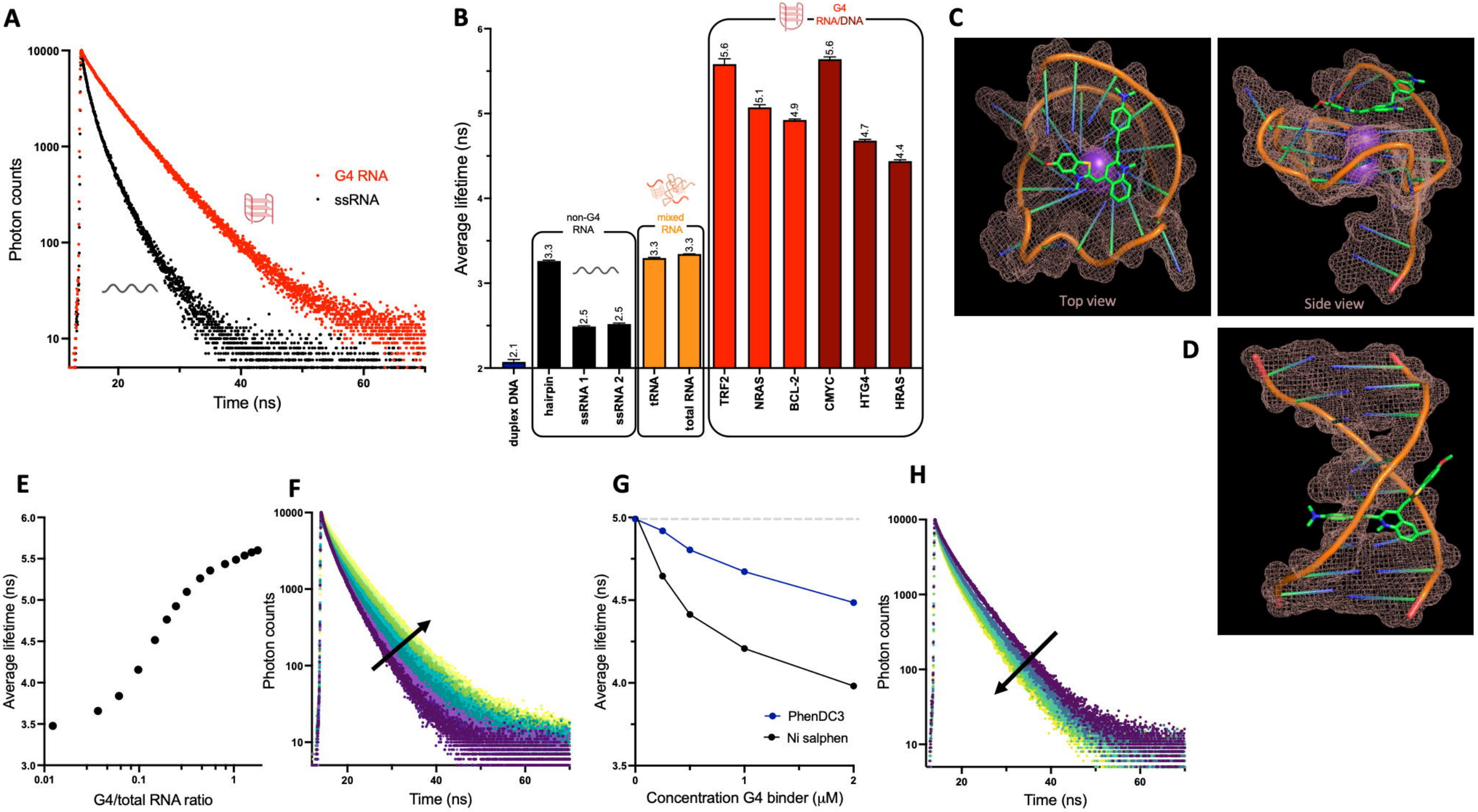
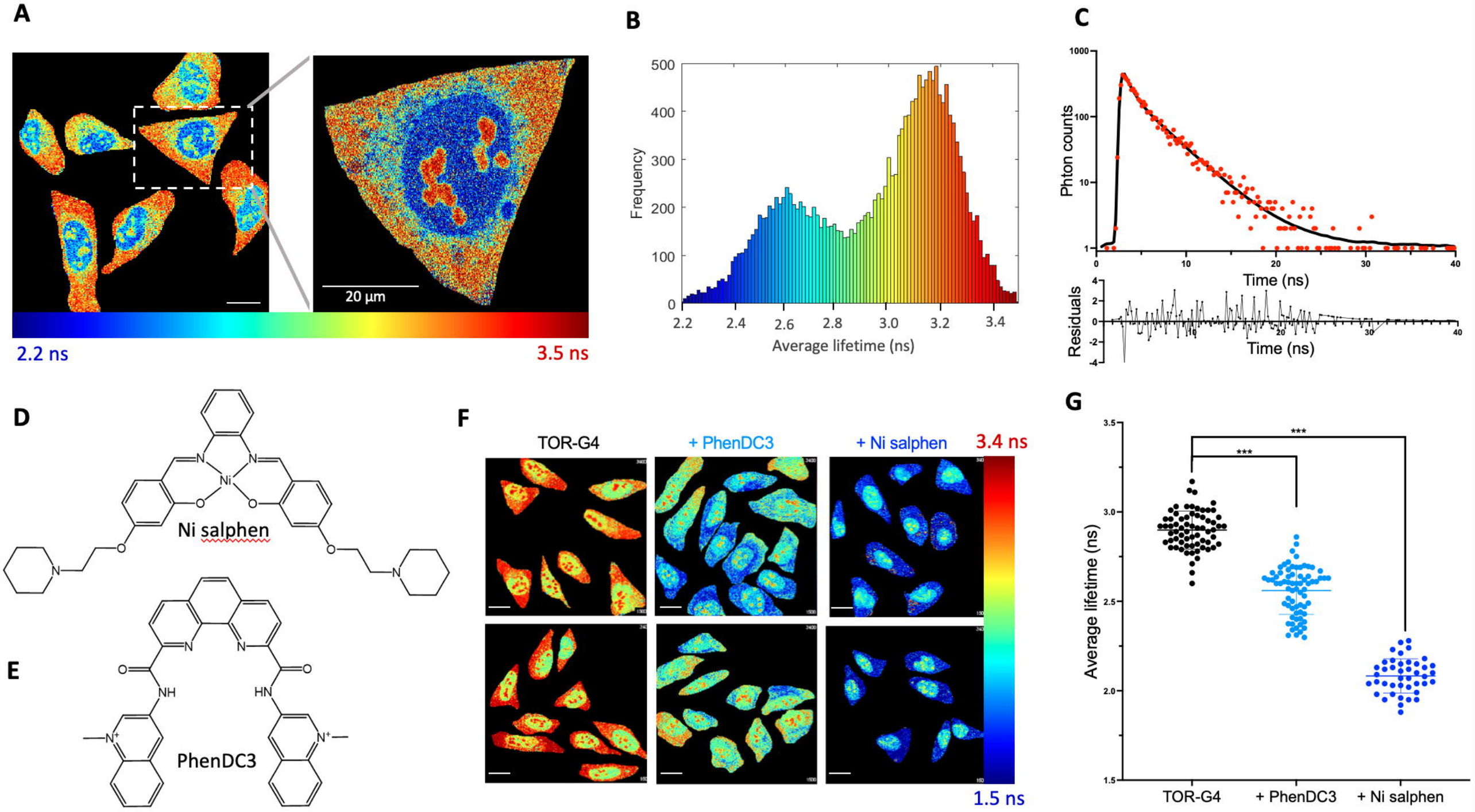
600
700
800
Wavelength (nm)


A
Photon counts
Time (ns)
G4 RNA
ssRNA
B
Average lifetime (ns)
non-G4 RNA
mixed RNA
G4 RNA/DNA
duplex DNA
hairpin
ssRNA 1
ssRNA 2
tRNA
total RNA
TRF2
NRAS
BCL-2
CMYC
HTG4
HRAS
2.1
3.3
2.5
2.5
3.3
3.3
5.6
5.1
4.9
5.6
4.7
4.4
C
Top view
Side view
D
E
Average lifetime (ns)
G4/total RNA ratio
F
Photon counts
Time (ns)
G
Average lifetime (ns)
Concentration G4 binder (μM)
PhenDC3
Ni salphen
H
Photon counts
Time (ns)


A
20 µm
2.2 ns
3.5 ns
B
Frequency
Average lifetime (ns)
C
Phton counts
Time (ns)
Residuals
Time (ns)
D
Ni salphen
E
PhenDC3
F
TOR-G4
+ PhenDC3
+ Ni salphen
3.4 ns
1.5 ns
G
Average lifetime (ns)
TOR-G4
+ PhenDC3
+ Ni salphen