**G-quadruplexes: prediction, characterization, and biological application**

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**Abstract**

 Guanine (G)-rich sequences in nucleic acids can assemble into G-quadruplex structures that involve G-quartets linked by loop nucleotides. The structural and topological diversity of G-quadruplexes have attracted great attention for decades. Recent methodological advances have advanced the identification and characterization of G-quadruplexes *in vivo* as well as *in vitro*, and at a much higher resolution and throughput, which has greatly expanded our current understanding of G-quadruplex structure and function. Accumulating knowledge about the structural properties of G-quadruplexes has helped to design and develop a repertoire of molecular and chemical tools for biological applications. This review highlights how these exciting methods and findings have opened new doors to investigate the potential functions and applications of G-quadruplexes in basic and applied biosciences.

**Importance of G-quadruplex structures in biology**

Nucleic acid structures are fundamental to the cellular function and regulation of diverse biological events [1]. DNA and RNA sequences can fold into myriad structural motifs such as duplexes, hairpins, triplexes, pseudoknots and G-quadruplexes, to assemble the functional structural conformation for their precise biological roles in specific cellular environments [1]. Interestingly, Guanine (G)-rich sequences can self-associate into stacks of G-quartets (**Figure 1A**) to form complex structural motifs known as G-quadruplexes [2] (**Figure 1B**). G-quadruplexes are of growing interest in chemistry and biology, largely due to their peculiar and diverse molecular structures, which include **parallel** and **antiparallel** **topologies** (see glossary) (**Figure 1B**). Recently, G-quadruplexes have been reported to have critical regulatory roles in biological processes, including but not limited to DNA replication, transcription, and translation [3, 4] (**Figure 1C-E**), providing new and important mechanisms for controlling gene expression and genome stability. By understanding the principles of how G-quadruplex structures mediate gene expression in cells, one can harness their chemical and biochemical properties to aid in developing novel biological applications.

In this review, we first illustrate how G-quadruplexes can be used in biological applications. Next, we present the classical methods used to predict and identify G-quadruplex structures. Then, we highlight new molecular and chemical tools that enable detection of G-quadruplexes in cell imaging, followed by innovative next-generation sequencing techniques that map G-quadruplex structures on a genomic or transcriptomic scale. Novel biological insights that have resulted from these studies, and the current limitations of these methods, are discussed. Lastly, we present our perspectives on future advances and challenges toward a more complete understanding of G-quadruplex structure-function relationships *in vivo*,which will facilitate the potential development of a new set of G-quadruplex-based biological applications.

**G-quadruplexes as molecular tools for biological applications**

Being versatile in nature, G-quadruplexes have been identified on many occasions for the development of molecular tools binding to diverse classes of targets (**Table 1**). It is desirable to use G-quadruplex-containing aptamers as therapeutic and diagnostic agents for diseases such as cancers [5-7], as they are thermodynamically and chemically stable, resistant to many serum nucleases, and have a low immunogenicity and good cellular uptake [8].

Among the G-quadruplex-containing **aptamer**s reported to recognize proteins and enzymes (**Table 1**), one of the well-studied examples is thrombin-binding aptamer (TBA) [9], identified by an *in vitro* selection process called **SELEX** (**Figure 2A**), which binds to the exosite I of human thrombin with high affinity and selectivity. The crystal structure of TBA revealed that the DNA G-quadruplex is in anti-parallel topology with 2-quartet planes [10], and data suggested that the presence of thrombin induces the TBA to fold into a G-quadruplex conformation for binding [11]. Recent studies on TBA and variants suggest that they can be used for biosensing, with nanomolar affinity to thrombin [12, 13].

Besides proteins and enzymes, specific G-quadruplex-containing aptamers have also been found to recognize small molecules (**Table 1**). Using SELEX, an aptamer called “spinach” was identified that recognizes 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI), and produces fluorescence upon ligand binding [14]. Recent crystal structural studies showed that the ligand stacked with the unique G-quadruplex  [15] (**Figure 2B**). Notably, the G-quadruplex was found to be in a special structural scaffold in the spinach aptamer that is essential for the ligand binding and fluorescence [15]. Since then, the spinach aptamer has been modified and applied for live cell imaging to track different biomolecules [16, 17]. In addition, the sequence requirement for the fluorescence of spinach RNA aptamer was extensively studied [18], and several other fluorescent RNA aptamers, such as “mango” aptamer and other variants of “spinach”, have been shown to contain G-quadruplex structures [19-22], providing structural insights for future design and development of brighter fluorescent RNA for imaging purposes.

In one particularly versatile application of G-quadruplexes as biosensors, the G-quadruplex-containing aptamer PS2.M, initially selected via SELEX, was shown to possess catalytic properties upon binding with hemin [23] (**Figure 2C**). This G-quadruplex-hemin complex can be used to mimic the activity of horseradish peroxidase (HRP) [23] by catalytically oxidizing colorimetric substrates such as 3,3’,5,5’-tetramethylbenzidine (TMB) and 2,2’-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) upon hydrogen peroxide addition (**Figure 2C**). The sequence and structure requirements for the catalytic properties were tested comprehensively to verify the important role of the G-quadruplex in this peroxidase-mimicking system [24-27], which has since been developed into numerous applications including the detection of cations, organic molecules, proteins, nucleic acids, and others [28].

Another notable G-quadruplex application is quadruplex priming amplification (QPA). The strategy involves the spontaneous dissociation of DNA duplex and formation of DNA G-quadruplex upon primer extension reaction. The G-quadruplex is then detected via fluorescence signal from 2-aminopurine (2AP) (**Figure 2D**) [29], which substitutes the T at the first loop of the dG3T G-quadruplex, giving a fluorescence increase upon G-quadruplex formation. The QPA can be employed for both linear [29] and exponential [30] signal amplification modes to detect target sequences of interest. For example, QPA can be coupled with linear nicking amplification (LNA) [31], which allows detection of target sequences as low as the 10 fM [32]. This approach paves the way to detect low-abundance nucleic acid molecules, such as pathogenic DNA, for diagnostic applications. In QPA, the readout is fluorescence signal from 2AP or 3-methylisoxanthopterin (3MI); however, it might be interesting to see if it can be performed without the need of exogenous fluorophores, as several recent studies have reported the intrinsic fluorescence of the G-quadruplex in DNA and RNA [33-38].

**Predicting G-quadruplexes using computational methods**

 Classically, a nucleic acid sequence containing four runs of at least three guanines, separated by short stretches of other bases, can potentially fold into an intramolecular G-quadruplex, so the potential to form these motifs can be predicted from primary sequence. In an **intramolecular G-quadruplex** the guanine runs all occur on the same strand of DNA, whereas in an **intermolecular G-quadruplex** they occur on both the sense and antisense strands (**Figure 1B**). For intramolecular quadruplexes, many algorithms have been published over the past decade to predict the potential formation of G-quadruplexes directly from DNA sequence, including QuadParser  [39], QGRS mapper  [40], G4P Calculator  [41], QuadBase  [42] and most recently, G4 Hunter  [43]: their relative features are reviewed in  [44]. Some of these algorithms simply seek sequences bearing four tracks of three guanines in close proximity; others take into account additional factors that can influence G4 folding (discussed below) such as the length and nature of loops between the tracks of guanine. Predicting intermolecular G-quadruplexes is somewhat more complex because it requires consideration of both DNA strands  [45, 46], and algorithms have also been developed specifically for RNA [47] which, being single-stranded, can adopt myriad competing conformations besides G-quadruplexes  [48].

 Using such algorithms, it is of theoretical interest to predict how many putative G-quadruplex sequences (PQSs) a genome would be expected to contain at random – and therefore whether these sequences are over- or under-represented in real genomes. However, this remains a non-trivial problem because any simple model is rendered highly inadequate by variable genome composition (i.e. the overall percentage of guanines in a genome may not be distributed equally throughout) and by biased base dyad frequencies (i.e. the non-random likelihood that any G will be followed by a G, C, T or A)  [39, 49].

 The consensus sequence for PQSs has traditionally been G3 N1-7 G3 N1-7 G3 N1-7 G3  [39] but it is increasingly recognised that this consensus does not accurately predict all the PQSs in a genome  [50]: motifs with larger loops, non-guanine bulges, etc. may also form G-quadruplexes. Loops as large as N=30 can support G-quadruplex formation *in vitro*  [51] and most predictive algorithms permit a user-defined loop length. Short loops are, however, a major factor in G-quadruplex stability  [52, 53] and some algorithms incorporate this, together with other factors, into a sliding score for G-quadruplex propensity and stability, rather than a binary prediction  [41, 43, 54]. The picture is further complicated by recent evidence that ‘bulged’ G-quadruplexes can occur *in vitro* (with a non-guanine base interrupting a 3-guanine track sequence)  [50, 55], and also that two instead of three guanine quartets can suffice, particularly in RNA, giving rise to G2 Nx quadruplexes  [56]. Since no predictive algorithm is perfect for all purposes, users must define their parameters appropriately and balance the chances of false positives against false negatives. It is then important to confirm *in silico* predictions via the *in vitro* and/or *in vivo* methods discussed below.

**Identifying and characterizing G-quadruplexes using biophysical and biochemical methods**

A number of experimental methods have been developed to support the computational prediction of G-quadruplexes. These methods can be broadly defined into 2 classes: biophysical and biochemical methods (**Table 2**).

Thanks to the unusual structure and folding of the G-quadruplex, one can experimentally identify G-quadruplex formation and investigate structural properties using biophysical techniques (**Table 2**). For example, the topology of the G-quadruplex structure can be determined by monitoring the positive or negative circular dichroism (CD) signals at specific wavelengths [57]. In general, G-quadruplexes with parallel topology (**Figure 1B**) have negative and positive CD signals at 240 nm and 262 nm respectively, whereas anti-parallel topology (**Figure 1B**) places these signals at 262 nm and 295 nm respectively. To verify G-quadruplex formation, one should also perform the CD experiments under non-G-quadruplex stabilizing (Li+) and G-quadruplex stabilizing conditions (such as K+ or with G-quadruplex ligands), and scan towards the far-UV region (~180-230nm). Likewise, the thermostability of the G-quadruplex structure can be identified by observing the ultraviolet (UV) signal at 295 nm [58]. Upon G-quadruplex melting, the UV absorbance at 295 nm decreases, leading to a hypochromic shift that is a distinctive feature of G-quadruplex structure. In addition, G-imino protons involved in G-quartets will exhibit a distinct proton chemical shift value of 10.5-12 ppm in NMR. Moreover, certain dyes such as the benzothiazole Thioflavin T (ThT) and N-methyl mesoporphyrin IX (NMM) have been found to fluoresce upon binding to G-quadruplexes [59, 60], providing **light-up** structural probes to detect G-quadruplexes *in vitro*: such chemical tools were recently reviewed [61]*.* These and other biophysical techniques (**Table 2**) are widely used under different *in vitro* conditions to verify G-quadruplex formation; however, these methods are limited to studying short oligonucleotides and thus do not account for the effect of flanking sequences.

To address this issue, biochemical techniques were employed to interrogate G-quadruplex formation in a longer sequence context (**Table 2**). In the DNA polymerase stop assay, the formation of a G-quadruplex in a DNA template can act as a roadblock and cause polymerase stalling, which halts the primer extension. Salazar and colleagues previously applied this method to study the DNA G-quadruplex structure formed by telomeric DNA sequences, d(T2G4)4 or d(T2AG4)4, in the template strand [62]. The dimethyl sulfate (DMS) followed by the piperidine cleavage assay is based on the fact that the formation of a G-quadruplex will prohibit the N7 guanine methylation caused by DMS, leading to a protection pattern observed at the DNA G-quadruplex region after piperidine cleavage. For example, Cech and colleagues used this technique to interrogate telomeric DNA sequences and observed such DMS protection pattern in the G-quadruplex site [63]. In-line probing (ILP) is a slow, spontaneous RNA cleavage reaction that measures the flexibility of each RNA nucleotide: this method was first developed to study the structure of riboswitches, and later applied to RNA G-quadruplexes [64]. Several recent studies have reported the use of ILP to probe the formation of G-quadruplexes in messenger RNAs [48, 65].

Recently, several new biochemical methods were developed to study RNA G-quadruplexes (**Table 2**). Reverse transcriptase can be stalled by RNA G-quadruplex structures during reverse transcription. Kwok and Balasubramanian developed a reverse transcriptase stalling (RTS) assay and coupled this with ligation-mediated PCR to identify the *in vitro* G-quadruplex formation in low-abundance human telomerase RNA [66]. RNA structure can be probed by chemical probes such as DMS and **SHAPE** reagents [67, 68], and analysed by primer extension. The same group reported the novel use of lithium-based primer extension (LiPE) buffer in reverse transcription that alleviates RTS, and coupled it with SHAPE reagents and DMS to develop SHALiPE and DMSLiPE [69]. Application of *in vitro* SHALiPE and DMSLiPE has revealed G-quadruplex formation in precursor microRNA 149 [69]. Dominguez and coworkers developed a method called FOLDeR (footprinting of long 7-deazaguanine-substituted RNAs), which compared the RNase footprinting results between wildtype and 7-deazaguanine-substituted RNA [70]. Results from FOLDeR have revealed the *in vitro* formation and location of RNA G-quadruplexes in a 681-nucleotide fragment of Bcl-x RNA [70].

Most of the biophysical and biochemical assays described here (**Table 2**) are limited to *in vitro* studies; however, several methods can be adapted for *in vivo* applications, such as the DMS and piperidine cleavage assay, SHALiPE and DMSLiPE. Complementing these exciting biochemical methods with functional assays (e.g. reporter genes, western blotting, RNA processing assays) and cell imaging experiments (as discussed below) will enable us to uncover the structural and functional role of G-quadruplexes in cells.

**Visualizing G-quadruplexes using cell imaging methods**

Methods for detecting G-quadruplexes in whole cells have advanced significantly in recent years (**Table 3**). Two structure-specific antibodies are now available to facilitate G-quadruplex immunofluorescence in a range of eukaryotic cells  [71, 72] and in cells infected with G-quadruplexes-rich viruses  [73]. In parallel, a range of ‘light-up’ chemical probes is rapidly being developed, with the potential advantage over antibodies that they could be deployable in living cells.

 The first quadruplex-specific antibody, Sty49, was used over a decade ago to visualise G-quadruplexes in ciliate macronuclei  [74], where large amounts of telomeric DNA offer a super-abundance of G-quadruplexes. A long delay then ensued before successful detection was reported in fixed mammalian cells (where telomere repeats are much less abundant). Whole-cell immunofluorescence assays may be particularly challenging because chromatin can obscure G-quadruplex epitopes, and because G-quadruplexes may be dynamic and fold only transiently *in viv*o. Several different protein probes were engineered, including a zinc-finger protein GQ1 [75], a range of designed ankyrin repeat binding proteins or ‘DARPins’  [76] and a single-chain antibody hf2  [77]: these could all detect G-quadruplexes *in vitro* but proved unsuitable for whole-cell immunofluorescence  [76]. Since hf2 could be used to pull-down G-quadruplex DNA fragments from purified genomic DNA  [78], the primary problem was probably not the sensitivity of these tools, but the *in cellulo* chromatin context.

Two new antibodies, BG4  [71] and 1H6  [72], have recently proved suitable for whole-cell immunofluorescence on fixed mammalian cells, although questions remain about whether they can be sensitive to the level of a single G-quadruplex, or can only detect high local densities of multiple G-quadruplex motifs. The number of BG4 foci detected in fixed human cells is orders of magnitude lower than the number of G-quadruplex-forming sequences predicted throughout the genome, but dynamic and transient folding might be expected to limit detection *in vivo*, and most antibodies will not detect every possible structural variant of G-quadruplex. Indeed, although the two antibodies were generated via different routes, 1H6 by immunizing mice with stable G-quadruplex structures and BG4 by phage display and *in vitro* selection on such structures, both methodologies are highly dependent upon the exact structures chosen as antigens. Concerning RNA G-quadruplexes, 1H6 apparently does not detect these  [72] whereas BG4 does  [79].

 Turning to the development of small-molecule probes for G-quadruplexes, the status of this field has been recently reviewed  [61, 80, 81] and several representative probes are presented in **Table 3**. In brief, probes for use in living cells should be membrane-permeable and minimally cytotoxic, as well as highly selective for G-quadruplex motifs, with strong and specific ‘light-up’ (or ‘**light-off**’) emission versus minimal background. Furthermore, they should not actually induce G-quadruplex formation, but simply detect pre-existing G-quadruplexes – a difficult distinction to assess. This combination of criteria is very demanding and no perfect probe has yet been reported. However, there are many promising candidates, including sensors for DNA  [82, 83], RNA  [84, 85] and both DNA and RNA G-quadruplexes  [86-88], based on a wide variety of chemistries and possessing combinations of properties (e.g. non-toxicity, cell permeability, G4-induction, specificity or non-specificity for particular structural topologies) whose relative importance must be evaluated for particular applications. Certain probes may be targeted to a particular G-quadruplex-encoding sequence by conjugation with a gene-specific oligonucleotide  [85]. Another approach is to attach a fluorophore to a known G-quadruplex-binding protein, thus circumventing the inherent tendency of guanines to quench fluorescence from small molecules, and potentially also mitigating cytotoxicity. A G-quadruplex-binding peptide from the RHAU helicase has recently showed promise as a sensor *in vitro*  [89]. Importantly, all such probes will be subject to the same unanswered question as antibodies concerning their sensitivity: can a single G-quadruplex motif ever be detected *in cellulo*? As an ultimate goal for this field, such spatial (and, in live cells, temporal) resolution could be enormously powerful, allowing researchers to determine where and under what cellular conditions particular G-quadruplexes can fold, and how they might respond to changing conditions such as transcriptional stimuli, DNA damage, or different cell cycle phases.

**Genome/Transcriptome-wide mapping of G-quadruplexes using sequencing methods**

In contrast to the above-described challenge of detecting single G-quadruplex motifs via whole-cell imaging, the advent of next-generation sequencing (**NGS**) has provided an excellent opportunity to design G-quadruplex-specific NGS methods at a genome/transcriptome-wide level. They can be broadly categorized into two approaches: the antibody-mediated pull-down approach, and the polymerase stalling approach.

 For the antibody-mediated pull-down approach, successful chromatin immunoprecipitation (**ChIP**) of G-quadruplexes was reported only very recently – possibly because suitable antibodies proved elusive, because the native chromatin context tends to mask the majority of G-quadruplex epitopes, and/or because the PQSs are folded into G-quadruplexes only in specific cellular conditions. Circumventing some of these issues, an antibody pull-down was conducted from naked genomic DNA using the hf2 antibody (**Figure 3A**) but this detected only ~700 G-quadruplexes [78]; subsequently the BG4 antibody (which was also used in a chromatin context for whole-cell IFAs) was used to isolate G-quadruplex-containing fragments from human chromatin, yielding ~10,000 or 1,000 motifs from two different human cell lines (**Figure 3B**)  [90]. The isolated regions tended to be non-coding regulatory regions of highly-transcribed genes, suggesting that nucleosome depletion and active transcription probably favour the folding of G-quadruplexes [90]. In a related ‘indirect ChIP’ approach [91], sites of DNA damage marked by the histone variant H2AX were pulled down after treating cells with the quadruplex-stabilizing drug pyridostatin (PDS), on the hypothesis that persistent quadruplexes would induce transcription-and/or replication-dependent DNA damage. This yielded large sequence domains because the histone mark spreads quite broadly at sites of damage, but the domains were indeed enriched in putative quadruplex-forming sequences [91].

 Polymerase stalling approaches such as G4-seq offer, by contrast, the comprehensive experimental identification of sequences that *can* form G-quadruplexes (**Figure 3C**) [50], yielding an *in vitro* genome-wide G-quadruplex map. Here, sheared DNA is subjected to NGS in the presence or absence of conditions that favour quadruplex folding (potassium ions and/or the G-quadruplex-stabilising ligand pyridostatin). Under stabilising conditions, G-quadruplexes impede the polymerase, causing a characteristic increased mutation rate in sequence data at the G-quadruplex folded region. The G4-seq technique identified ~700,000 G-quadruplexes in the human genome: orders of magnitude more than ChIP and twice the number predicted *in silico* by standard algorithms. This is because many of the sequenced motifs were bulged or long-looped – and indeed, the majority of G-quadruplexes found by ChIP (79%) were not of the canonical G3 N1-7 type either [52]. However, those that *were* of this type tended to represent the strongest ChIP peaks, and there was also a reassuring degree of consonance between the ChIP and G4-seq experiments: 87% of the DNA fragments from ChIP contained sequences identified in G4-seq [50].

 The difference in the number of G4s reported from datasets obtained *in vitro* and *in vivo* (i.e. G4-seq versus G4-ChIP) suggests that the cellular environment may play a central role in affecting the dynamics of G-quadruplex formation in cells. Specifically, many cellular factors, such as G-quadruplex binding proteins and helicases (**Box 1**), can likely remodel the DNA G-quadruplex landscape *in vivo*. Nevertheless, more technical explanations for the difference in G-quadruplex detection rates *in vivo* versus *in vitro* cannot be excluded, such as limited sensitivity of ChIP and condition-dependence for G-quadruplex formation in native chromatin. Results are also likely to be influenced by sensitivity/specificity of the antibody used, choice of cell line, and variation in experimental protocols and bioinformatics pipelines. Future development in G-quadruplex-antibody-based sequencing methods should aim to address these issues in order to establish a gold standard for robust mapping of DNA G-quadruplexes in native chromatin for different species.

Turning from DNA to RNA G-quadruplexes, a polymerase stalling approach called rG4-seq was recently reported (**Figure 3D**)  [56], based on the working principle first developed in RTS (described above) [66]. Here, RNA G-quadruplexes impede the reverse transcriptase enzyme used to generate an NGS library from polyA-enriched RNAs. rG4-seq is particularly useful for generating an *in vitro* transcriptome-wide map of RNA G-quadruplexes, which will serve as a useful guide for future *in vivo* studies. Application of rG4-seq has identified a preponderance of unconventional G-quadruplexes such as long loops, bulged, and 2-quartet structures. Notably, significant correlations were reported between RNA G-quadruplexes and cis-regulatory elements such as microRNA target sites and polyadenylation signals. Further in-depth studies may examine how RNA G-quadruplexes may regulate microRNA targeting and alternative polyadenylation to affect gene expression and RNA metabolism in cells.

As discussed above and shown in **Table 2** (see DMSLiPE and SHALiPE), chemical-based RNA structure probing assays  [69] have the advantages to probe *in vivo* RNA structure on a transcriptome-wide scale [67, 68, 92]. A recent report from Guo and Bartel used DMS and SHAPE chemical probing and reported that RNA G-quadruplexes are under-represented in bacteria, and are globally unfolded in human, mouse and yeast, suggesting that eukaryotic cellular machinery may actively control or unwind RNA G-quadruplexes [93]. These results advance our understanding of the dynamic (un)formation and role of RNA G-quadruplexes in cells, as many thousands G-quadruplexes were reported to form *in vitro* [56, 93], yet less so *in vivo* [93]. Future experiments may investigate whether these phenomena are generally applicable to other cell types, cellular conditions and species by using multiple RNA structurome and interactome methods [67, 68, 92], and orthogonal G-quadruplex antibody-mediated pull down approaches as similarly performed for DNA G-quadruplexes. One future challenge is to identify and characterize the effect of known and unknown RNA G-quadruplex binding proteins (**Box 1**) on G-quadruplex structure and function. Overall, these genome- and transcriptome-wide studies generate new testable hypotheses and offer future directions to explore the G-quadruplex-mediated biological processes across the tree of life.

**Future perspectives and challenges**

Amongst the technologies discussed in this review, some are quite advanced while others remain in their infancy. Biophysical – and to some extent, biochemical – methods for identifying G-quadruplexes have been developed and applied for many years and a wealth of *in silico* algorithms is available for predicting G-quadruplex formation from nucleic acid sequences. However, it is increasingly clear that the rules for G-quadruplex folding are complex, subtle and context-dependent. Few of the algorithms comprehensively incorporate empirical experimental data, few biophysical techniques incorporate the wider sequence context, and few biochemical techniques are applicable *in vivo* to date (**Table 2**). As such, the field calls for new *in vivo* methods with superior resolution, throughput, and sensitivity to investigate the spatial-temporal formation of G-quadruplexes, their structure folding and dynamics, and the effect of cellular factors upon G-quadruplexes that prevail in cellular milieu.

Cell imaging methods have advanced tremendously in the past few years, but challenges remain around the feasibility of resolving single G-quadruplex motifs, the relative accessibility of G-quadruplexes in chromatin contexts, and the potentially transient nature of many G-quadruplexes *in vivo*. Also, it is yet to be seen if the G-quadruplex-specific probes (**Table 3**) can be easily applied to other biological systems. Notably, the cellular localization and live cell imaging of G-quadruplexes in particular genes is largely untested: this would require sequence-specific G-quadruplex antibodies, oligonucleotide-conjugated G-quadruplex probes or a combination of IFA and FISH (both of which are highly demanding in terms of sensitivity). In addition, the production of the perfect light-up G-quadruplex probes for use in living cells remains elusive, although some recent progress has been made towards this goal [61, 80, 81].

Like whole-cell imaging, the ‘omic’-level sequencing techniques now available have exploded recently with a series of seminal papers. In this field, defining the formation, structure dynamics, and interaction partners of G-quadruplexes *in cellulo* remains the key question. G-quadruplex formation can be influenced by a variety of factors, including metal ions, flanking sequence context, and proteins. Recent studies showed that G-quadruplexes could interconvert with stem-loop structures to regulate cellular processes [69, 94-96], suggesting that alternative structures such as duplexes or hairpins may compete with G-quadruplex formation *in vivo*. It will be interesting to structurally probe them *in vivo* and see how prevalent these G-quadruplex structure-switches are, and what are their regulatory roles *in vivo*. As mentioned above and detailed in **Box 1**, some known G-quadruplex binding proteins and helicases have been identified; however, their global effects on G-quadruplex structures in cells are largely uncharacterized. In the future, further studies performed under knock-down/knock-out of G-quadruplex binding proteins, and under normal and stress conditions, could provide clues about the formation, structure dynamics, and interactions of G-quadruplexes *in vivo.*

Most G-quadruplex studies consider only intramolecular G-quadruplex folding; however, bioinformatics searches have shown the potential prevalence of intermolecular DNA:RNA G-quadruplexes in humans [97]. Given the vast number of predicted intermolecular G-quadruplexes, great experimental effort and robust analysis platforms are needed to reveal their pervasiveness, their structural conformational exchange with intramolecular G-quadruplexes or other structural motifs, and their potential functions in cells, such as in transcription. Innovative strategies are thus urgently needed to be able to detect and characterize these intermolecular G-quadruplex motifs *in vivo*.

**Concluding remarks**

Remarkable progress has been made in G-quadruplex research in the past 5 years. This is an exciting time to explore the *in vivo* G-quadruplex structure at unprecedented resolution, throughput, and sensitivity. We are cautiously positive that the development of a suite of novel methodologies will reveal the *in vivo* structures and functions of G-quadruplexes in diverse organisms, and help to address the biological questions regarding the prevalence, location, diversity, dynamics, interactions, and localization of G-quadruplexes in diverse organisms (see Outstanding Questions). As emerging evidence suggests a connection between G-quadruplexes, gene regulation, and development of diseases [98-100], these upcoming advancements in G-quadruplex research will decipher the underlying biochemical mechanism and the molecular basis of diseases, and also facilitate the rational design and development of G-quadruplex-related tools for various biological applications. We look forward with great optimism to the next set of groundbreaking discoveries and applications to be unveiled in the next 5 years.

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**Figure 1.** **G-quadruplex structure and biology.** A) Chemical structure of a G-quartet. Potassium ion (K+) sits within the G-quartet for stabilization. G-quartets stack on each other to form G-quadruplex. B) Representative topologies of G-quadruplex structures. C-E) Representative G-quadruplex-associated biology: regulation of C) DNA replication, D) transcription, E) translation.

**Figure 2.** **G-quadruplexes as molecular tools for biological applications.** A) Schematic representation of SELEX. Random single-stranded DNA or RNA oligonucleotides are synthesized, and are subjected to interaction with the target of interest in the selection step. After that, the washing step removes the unbound oligonucleotides and retains the tightly bound ones. These bound oligonucleotides are then eluted out and are amplified by PCR (for ssDNA) or RT-PCR (for ssRNA). After several cycles, the final candidates are cloned and sequenced to identify the DNA or RNA sequence. B) The G-quadruplex domains of Spinach RNA aptamer (PDB ID:4KZD). The figures were adapted and modified from [15]. C) Schematic representation of hemin/G-quadruplex Horseradish Peroxidase (HRP)-mimicking DNAzyme. G-rich sequences such as PS2.M can fold into a G-quadruplex in the presence of K+ ion, which will then bind to hemin. The hemin-quadruplex complex possesses peroxidase properties that can catalyse the conversion of hydrogen peroxide to water. Colorimetric substrates such as TMB or ABTS can be used to monitor the process. D) Schematic representation of QPA. The 2AP containing G-rich primer strand (red) that is missing one track of Gs required to form a G-quadruplex binds with the C-rich template strand (blue). Addition of DNA polymerase allows the missing Gs to be filled in, causes duplex dissociation and induces G-quadruplex formation. The formation of the G-quadruplex leaves the 2AP to be unstacked at the loop of the G-quadruplex, thus producing fluorescence signal for detection.



**Figure 3.** **Schematics of G-quadruplex-specific NGS methods.** A) hf2 G4 pull-down sequencing [78]: Genomic DNA was isolated from cultured cells and fragmented by sonication. The fragmented DNA was then incubated with G4-specific hf2 antibody. The mixture was washed to remove hf2-unbound DNA, and the hf2-bound DNA was subsequently eluted. The recovered DNA was then library prepared for NGS. B) BG4 ChIP-seq [3]: Cells were fixed with formaldehyde, then chromatin was isolated and fragmented by sonication. The fragmented chromatin was incubated with G4-specific BG4 antibody with a FLAG-tag. The mixture was washed to remove BG4-unbound chromatin, and the BG4-bound chromatin was subsequently eluted. The recovered DNA was library prepared for NGS. C) G4-seq [50]: Genomic DNA was isolated and fragmented by sonication. The fragmented DNA was then library prepared for NGS. The template DNA was first sequenced under Na+-containing condition to yield read 1. The newly synthesized strand was denatured and washed away. The template DNA was renatured with fresh primer and sequenced again under K+-containing (or Na++PDS-containing) conditions to yield read 2. D) rG4-seq [56]: Transcriptomic RNA was isolated from cultured cells, polyA-RNA selected, and fragmented by hydrolysis. 3’-adapter ligation was performed to provide a handle for reverse transcription in the subsequent step. The ligated RNA was divided into three reactions (Li+-containing, K+-containing, K++PDS- containing) and renatured, followed by reverse transcription. The cDNA fragments were ligated to a 5’-adaptor, followed by PCR and NGS.

**Table 1. Representative list of G-quadruplex-containing aptamers.**

|  |  |  |  |
| --- | --- | --- | --- |
| **G4/rG4-containing aptamer** | **Targetsa** | **Aptamer sequences** | **References** |
| T40214 | STAT3 | d(GGGCGGGCGGGCGGGC) |  [101] |
| HJ24 | Shp2 | d(AGCGTCGAATACCACACGGGGGTTTTGGTGGGGGGGGCTGGGTTGTCTTGGGGGTGGGCTAATGGAGCTCGTGGTCAT) |  [102] |
| 3R02 | VEGF | d(TGTGGGGGTGGACTGGGTGGGTACC) |  [103] |
| ISIS 5320 | HIV gp120 | d(T\*T\*G\*G\*G\*G\*T\*T) |  [104] |
| AS1411 | Nucleolin | d(GGTGGTGGTGGTTGTGGTGGTGGTGG) |  [105] |
| 93del | HIV Integrase | d(GGGGTGGGAGGAGGGT) |  [106] |
| RT6 | HIV ReverseTranscriptase | d(ATCCGCCTGATTAGCGATACTCAGGCGTTAGGGAAGGGCGTCGAAAGCAGGGTGGGACTTGAGCAAAATCACCTGCAGGGG) |  [107] |
| ODN 93 | HIV RNase H | d(GGGGGTGGGAGGAGGGTAGGCCTTAGGTTTCTGA) |  [108] |
| ODN 112 | HIV Rnase H | d(CCAGTGGCGGGTGGGTGGGTGGTGGGGGGACTTGG) |  [108] |
| TBA | Thrombin | d(GGTTGGTGTGGTTGG) |  [9] |
| RA-36 | Thrombin | d(GGTTGGTGTGGTTGGTGGTTGGTGTGGTTGG) |  [109] |
| Scl 2 | Sclerostin | d(TTGCGCGTTAATTGGGGGGGTGGGTGGGTT) |  [110] |
| R12 | PrPC | r(GGAGGAGGAGGA) |  [111] |
| PPK2 G9 | PPK2 | d(AACACATAGGTTTGGTTAGGTTGGTTGGTTGAATTA) |  [112] |
| Spinach | DFHBI | r(GACGCAACUGAAUGAAAUGGUGAAGGACGGGUCCAGGUGUGGCUGCUUCGGCAGUGCAGCUUGUUGAGUAGAGUGUGAGCUCCGUAACUAGUCGCGUC) |  [14] |
| Mango | TO1 | r(UACGAAGGGACGGUGCGGAGAGGAGAGUA) |  [19] |
| PS2.M | Hemin | d(GTGGGTAGGGCGGGTTGG) |  [23] |

\* = phosphorothioate bond

a STAT3, Signal transducer and activator of transcription 3. VEGF, Vascular endothelial growth factor. HIV, human immunodeficiency virus. PrPC, cellular prion protein, PPK2, polyphosphate kinase. DFHBI, 3,5-difluoro-4-hydroxybenzylidene imidazolinone. TO1, thiazole orange.

**Table 2. Representative biophysical and biochemical methods to study G-quadruplexes**

|  |  |  |  |
| --- | --- | --- | --- |
| **Methoda** | **G-quadruplex information** | **Features and limitations** | **References** |
| CD | Topology | DNA and RNA, short oligonucleotide, not applicable in vivo |  [57] |
| UV melting | Thermostability | DNA and RNA, short oligonucleotide, not applicable in vivo |  [58] |
| FRET melting | Thermostabiliity | DNA and RNA, short oligonucleotide, not applicable in vivo |  [113] |
| NMR | G-quartet imino protons at 10.5-12.0ppm3D structure | DNA and RNA, short oligonucleotide, not directly applicable in vivo |  [114] |
| X-ray crystallography | 3D structure | DNA and RNA, short oligonucleotide, not applicable in vivo |  [115] |
| Fluorescent probes (e.g. NMM, ThT) | Presence/absence | DNA and RNA, short oligonucleotide, not applicable in vivo |  [59, 60] |
| TDS | Presence/absence | DNA and RNA, short oligonucleotide, not applicable in vivo |  [116] |
| Optical tweezer | Mechanism stability | DNA and RNA, short oligonucleotide, not applicable in vivo |  [117] |
| Polymerase stop assay | Starting location at nucleotide resolution | DNA, no information on loops and other G-tracks, not applicable in vivo |  [62] |
| DMS and piperdine cleavage assay | Location and structural reactivity at guanine nucleotide resolution  | DNA, applicable in vivo, no information on loops |  [63] |
| RNase T1 | Location and structural reactivity at guanine nucleotide resolution | RNA, no information on loops, not applicable in vivo |  [118] |
| In line probing | Location and structural reactivity at single nucleotide resolution | RNA, react with 2’OH, not applicable in vivo |  [64] |
| RTS | Starting location at nucleotide resolution | RNA, no information on loops and other G-tracks, not applicable in vivo |  [66] |
| SHALiPE (and DMSLiPE) | Location and structural reactivity at single nucleotide resolution | RNA, applicable in vivo, react with 2’OH |  [69] |
| FOLDeR | Location and structural reactivity at single nucleotide resolution | RNA, requires multiple RNases and 7-deazaguanine substitution, not applicable in vivo,  |  [70] |

aCD, circular dichroism; UV, ultraviolet; FRET, fluorescence resonance energy transfer; NMR, nuclear magnetic resonance; ThT, benzothiazole Thioflavin T and NMM, N-methyl mesoporphyrin IX; TDS, thermal difference spectra; DMS, dimethyl sulphate; RTS, reverse transcriptase stalling; SHALiPE, selective 2’hydroxyl acylation analysed by lithium-based primer extension; FOLDeR, footprinting of long 7-deazaguanine-substituted RNAs

**Table 3. Representative G-quadruplex-specific antibodies and chemical probes for cell imaging.**

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Antibodies/probes** | **Application to date** | **Features and limitations** | **References** | **Commercially available?** |
| GQ1 zinc-finger protein | *In vitro* detection of DNA G4s | Not applied in whole-cell IFA |  [75] | No, see  [75] for synthesis |
| G4 DARPins | *In vitro* detection of DNA G4s | Not successful in whole-cell IFA |  [76] | No, see  [76] for synthesis |
| hf2 single-chain antibody | *In vitro* detection of DNA G4s, pull-down of G4s from genomic DNA | Not applied in whole-cell IFA |  [77, 78] | No, see  [77] for synthesis |
| Sty49 single-chain antibody | *In vitro* detection of DNA G4s, IFA on fixed ciliate cells | Detects only high-abundance telomeric G4 DNA in macronuclei (fixed cells). |  [74] | No, see [74] for synthesis |
| BG4 single-chain antibody | IFA on human cells, DNA and RNA G4s | Requires 3-step antibody staining protocol. Sensitivity to single-G4 level unproven. Used on fixed cells. |  [71, 79]  | Yes  |
| 1H6 mouse monoclonal antibody | IFA on human cells, DNA G4s only | 2-step antibody staining protocol. Detect DNA not RNA G4s; cross-reaction recently reported with poly-T DNA. Sensitivity to single-G4 level unproven. Used on fixed cells. |  [72, 73, 119] | Yes  |
| 3,6-bis(1-methyl-4-vinylpyridinium) carbazole diiodide (BMVC) | DNA G4s in human cells | Light-up, cell permeable (i.e. live cells). Can induce G4s folding *in vivo* |  [82] | No, see  [120] for synthesis |
| squarylium dye TSQ1 | DNA G4s in human cells | Light-up, cell permeable (i.e. live cells). Does not induce G4s folding *in vivo* |  [83] | No, see  [83] for synthesis |
| cyanine dye CyT | RNA G4s in human cells | Light-up, cell permeable (i.e. live cells). |  [84] | No, see  [84] for synthesis |
| G-quadruplex-triggered fluorogenic hybridization (GTFH) probe, ISCH-nras1 | RNA G4 in 5' UTR of NRAS mRNA | Light-up, Gene-specific. Not cell-permeable, not sensitive to natural low RNA levels |  [85] | No, see  [85] for synthesis |
| Anthrathiophenedione dye (ATPD) | DNA and RNA G4s in human cells | Light-up, cell permeable (i.e. live cells).  |  [86] | No, see  [86] for synthesis |
| NaphthoTASQ (N-TASQ) | DNA and RNA G4s in human cells | Light-up, affinity-triggered by contact with G4s ('smart probe'). Wavelength incompatible with standard light microscopy |  [88] | No, see [121] for synthesis |
| triangulenium derivative DAOTA-M2 | DNA and RNA G4s | Light-up, cell permeable (i.e. live cells), minimal toxicity |  [87] | No, see [87] for syntehsis |
| Fluorophore-conjugated RHAU helicase peptide | DNA G4s in vitro | Peptide-based. Not yet tested *in vivo*. |  [89] | No, see [89] for synthesis |

IFA, immunofluorescence assay

**Box 1. Proteins that bind and/or metabolize G-quadruplexes**

Many cellular proteins have been identified that interact with DNA and/or RNA quadruplexes: many of these are listed in the G4IPDB database (http://bsbe.iiti.ac.in/bsbe/ipdb/index.php) and some of the key players are tabulated below. These proteins can be used as tools to probe the distribution and function of quadruplex motifs, as well as being subjects of intense study themselves.

DNA G-quadruplexes

Proteins partners of DNA G-quadruplexes include several groups of structure-specific helicases, such as PIF1, RECQ and FANCJ (recently reviewed in  [122]), the transcriptional helicase complemention group XPD/XPB [123] and various non-helicase proteins including nucleolin  [124], shelterin components like POT1 [125] and transcription factors like Sp1 and MAZ [126, 127]. Deficiencies in RECQ, FANCJ, and XP helicases are linked to rare human disease syndromes: Fanconi’s anaemia for FANCJ, Xeroderma Pigmentosum for XPD/XPB and Bloom’s, Werner’s and Rothmund-Thomson syndromes for three members of the five-member RECQ family. The diseases are generally characterized by chromosomal instability, telomere deficiency, cancer proneness, etc.: the expected phenotypes for cells that cannot resolve non-canonical DNA secondary structures like G-quadruplexes, and hence suffer high rates of DNA replication fork stalling. At a molecular level, ChIP has demonstrated that these helicases tend to associate with PQSs in the genome, particularly when cells are treated with G-quadruplex-stabilising drugs  [128], while in helicase-deficient cells, genes whose expression is deregulated likewise tend to contain PQSs  [129]. However, the correlation is not direct and exclusive because some of the helicases also target other structures such as hairpins and chicken-foot structures  [130]. Accordingly, ChIP experiments for PIF1 and RECQs may yield many more targets than direct G4-ChIP.

RNA G-quadruplexes

Protein partners of RNA G-quadruplexes include helicases such as RHAU (DHX36) DHX9, as well as non-helicase proteins like FMRP and Aven. For more details, please see recent excellent reviews [100, 131, 132]. RHAU is one of the most studied helicases for RNA G-quadruplexes. RHAU is shown to be involved in the maturation of human telomerase RNA (hTERC) by unwinding the RNA G-quadruplex at the 5’end of hTERC [133]. FMRP is an important protein that is responsible for fragile X syndrome, and is crucial for cognitive development. Binding assays and bioinformatics analysis of NGS data suggested that it interacts with RNA G-quadruplexes [134, 135]. Recently, a crystal structure revealed that it requires an RNA duplex-quadruplex junction for recognition [136]. Similar to DNA G-quadruplex binding proteins, RNA G-quadruplex binding proteins also target other structures such as triple helices  [135, 137, 138]. Thus, one should be cautious about the RNA-protein NGS data obtained on these RNA G-quadruplex binding proteins, as they likely also contain structural motifs that do not fold into G-quadruplexes.

**Table within Box 1: Representative G-quadruplex-interacting proteins**

|  |  |  |  |
| --- | --- | --- | --- |
| **Protein name/family** | **DNA or RNA quadruplexes** | **Role** | **Reference** |
| PIF1 | DNA | Structure-specific 5’-3’ helicase, interacts with telomerase & regulates telomere maintenance |  [139] |
| RECQ family | DNA | Structure-specific 3’-5’ helicases, act at non-canonical DNA structures, facilitate DNA repair and suppress recombination  |  [122] |
| BRIP1 (FANCJ) | DNA | Structure-specific 5’-3’ helicase (RAD 3 family), primarily involved in repair of DNA crosslinks |  [122] |
| ERCC2/3 (XPB/XPD) | DNA | Helicase subunits of the TFIIH transcription/repair factor, involved in nucleotide excision repair. |  [123] |
| Nucleolin | DNA | Nucleolar protein, controls rRNA gene transcription & assembly/export of ribosomes |  [124] |
| POT1 | DNA | Shelterin component, protects telomeres |  [125] |
| PARP-1 | DNA | poly(ADP-ribose)polymerase, ADP-ribosylates many chromatin proteins is involved in DNA repair |  [140] |
| DHX36 (RHAU) | RNA & DNA | RNA (and DNA) helicase with preference for RNA G-quadruplexes |  [133] |
| DHX9 | RNA | RNA helicase with preference for RNA G-quadruplexes |  [141] |
| FMRP | RNA | Binds to mRNAs and regulates association with polysomes  |  [134, 135] |
| Aven | RNA | Regulator of apoptosis. Also associates with DHX36 helicase and binds mRNA G-quadruplexes  |  [142] |
| HnRNP F | RNA | Pre-mRNA processing and translocation |  [143] |
| DDX21 | RNA | RNA helicase with affinity for RNA G-quadruplexes |  [144] |
| CNBP | RNA | Zinc finger protein controlling the translational efficiency of mRNAs |  [145] |
| EBNA1 | RNA | Viral protein involved in controlling replication of EBV virus  |  [146] |

Given in parentheses are alternative names or the names of disease complementation groups caused by mutations of the given genes.

**Glossary box**

**Aptamer:** A biological molecule – usually a peptide or oligonucleotide – that binds to a specific target such as a protein or small molecule. Oligonucleotide aptamers (which may form G-quadruplexes) can be generated by combinatorial nucleic acid library screening, SELEX experiment, and other methods.

**ChIP:** ‘Chromatin ImmunoPrecipitation’. A technique to locate proteins – and also DNA motifs such as G-quadruplexes – in native chromatin. Chromatin is formaldehyde-fixed, extracted from cells, fragmented and treated with an antibody to the entity of interest in order to isolate associated DNA fragments. These are then identified by sequencing (ChIP-seq) or by hybridization to a microarray (ChIP-on-chip).

**Intra- & inter-molecular G-quadruplex:** An intramolecular quadruplex is formed from a single DNA strand, which bears four runs of guanine residues in close proximity. An intermolecular quadruplex is formed from runs of guanines on more than one DNA strand, or from a hybrid of DNA and RNA strands. See **Figure 1B**.

**Light up or light off probe:** A ‘light up’ probe displays enhanced fluorescence upon binding to its target whereas a ‘light off’ probe undergoes fluorescence quenching.

**NGS:** ‘Next-Generation Sequencing’. Modern, high-throughput sequencing techniques such as Illumina, Ion Torrent and 454, all of which produce sequence data concurrently on a genomic/transcriptomic scale in the form of millions of short sequence fragments (usually <1kb).

**Parallel & anti-parallel G-quadruplex topology:** A parallel G-quadruplex has all the guanine-bearing strands in the same 5’/3’ polarity, necessitating linking by ‘propeller type’ loops that run top-to-bottom of the folded motif. In an anti-parallel quadruplex, the strands do not all have the same polarity, and thus the linking loops can be at the top or bottom of the folded motif. See **Figure 1B**.

**SELEX:** ‘Systematic Evolution of Ligands by EXponential Enrichment’. A technique for generating highly target-selective oligonucleotides with strong binding affinity from a library of random sequences via repeated rounds of binding to the target ligand, washing, elution, reverse transcription (for RNA aptamer), and PCR amplification.

**SHAPE:** ‘Selective 2′-Hydroxyl Acylation analyzed by Primer Extension. SHAPE is used to determine RNA secondary structures by treating RNA with an acylation reagent that selectively acylates the flexible (unpaired) nucleotides of the RNA at the 2’hydroxyl (2’OH) group. These modifications can stall reverse transcriptase and thus provide an electrophoresis-based or NGS-based readout of nucleotide reactivity, which can then be used to infer RNA structure.

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