Supplementary Information

Discovery of a New Fluorescent Light-Up Probe Specific to Parallel G-Quadruplexes

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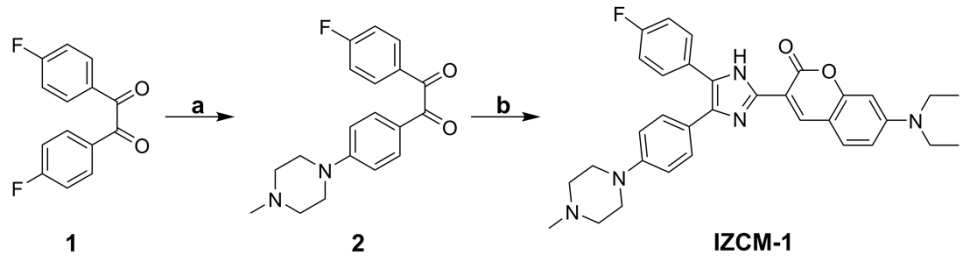
1. Experimental Section

1.1 Synthesis and Characterization

Scheme S1. Synthesis of **IZCM-1**[a]



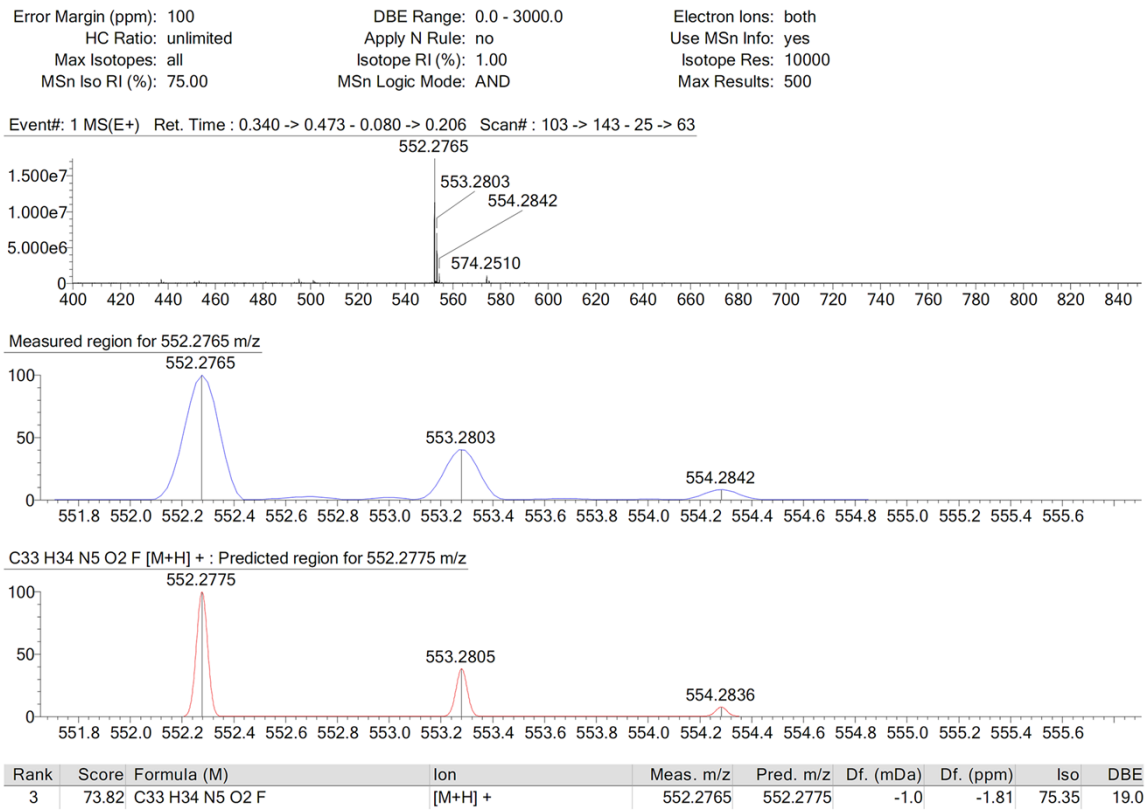
[a] Reagents and conditions: (a) N-Methylpiperazine, K_2CO_3, DMSO, 90 °C; (b) 7-diethylaminocoumarin-3-aldehyde, NH_4OAc, AcOH, reflux.

^1H and ^{13}C NMR spectra were recorded using TMS as the internal standard in methanol-d_4 or $CDCl_3$ with a Bruker BioSpin GmbH spectrometer at 400 MHz and 100 MHz, respectively. Mass spectra (MS) were recorded on a Shimadzu LCMS-2010A instrument with an ESI or ACPI mass selective detector and high resolution mass spectra (HRMS) on Shimadzu LCMS-IT-TOF. Melting points (m.p.) were determined using a SRS OptiMelt automated melting point instrument without correction. Flash column chromatography was performed with silica gel (200–300 mesh) purchased from Qingdao Haiyang Chemical Co. Ltd. The purity of synthesized compound was confirmed to be higher than 95% by using analytical HPLC performed with a dual pump Shimadzu LC-20 AB system equipped with a Ultimate XB-C18 column (4.6 × 250 mm, 5 μm) and eluted with methanol-water (65 : 35) containing 0.1% TFA at a flow rate of 1.0 mL/min. All chemicals were purchased from commercial sources unless otherwise specified. All the solvents were of analytical reagent grade and were used without further purification. Compound 7-diethylaminocoumarin-3-aldehyde was synthesized according to previous report.[1]

Synthesis of 1-(4-fluorophenyl)-2-(4-(4-methylpiperazin-1-yl)phenyl)ethane-1,2-dione (2).[2] To the solution of 1,2-bis(4-fluorophenyl)ethane-1,2-dione (1.48 g, 6.00 mmol) in DMSO (40 mL) was added N-Methylpiperazine (0.33 mL, 3.00 mmol) and K_2CO_3 (0.41 g, 3.00 mmol). The reaction mixture was stirred at 90 °C for 20 h, and was then extracted with EtOAc and water. The organic layer was dried over Na_2SO_4 and concentrated to give the compound **2** (1.31g, 67.0%): ^1H NMR (400 MHz, $CDCl_3$) δ 8.01 (dd, J = 8.8, 5.4 Hz, 2H), 7.85 (d, J = 9.0 Hz, 2H), 7.16 (t, J = 8.6 Hz, 2H), 6.87 (d, J = 9.1 Hz, 2H), 3.45 (d, J = 5.1 Hz, 4H), 2.55 (d, J = 5.1 Hz, 4H), 2.36 (s, 3H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 193.5, 192.0, 167.0, 155.1, 132.7, 132.3, 130.1,

122.5, 116.2, 113.2, 54.6, 46.8, 46.0. ESI-MS m/z: 327 $[M+H]^+$.

Synthesis of 7-(diethylamino)-3-(5-(4-fluorophenyl)-4-(4-(4-methylpiperazin-1-yl)phenyl)-1H-imidazol-2-yl)-2H-chromen-2-one (IZCM-1). A mixture of **2** (0.65 g, 2.00 mmol), 7-diethylaminocoumarin-3-aldehyde (0.61 g, 2.50 mmol), NH_4OAc (1.54 g, 20.00 mmol) and AcOH (13 mL) was stirred at reflux temperature for 2 h. After cooling, the mixture was treated with 3 mol/L HCl (aq) to reach the pH of 1, and washed by diethyl ether (10 mL×5) to remove the unreacted compounds. Then, the mixture was treated with 3.00 mol/L NaOH (aq) to reach the pH of 8, and the product was exacted by CH_2Cl_2 (20 mL×5). The combine organic phase was dried over Na_2SO_4 and solvent was removed by rotary evaporation. The crude product was purified by using flash column chromatography with $CHCl_3$/ CH_3OH (50 : 1) elution to afford an orange solid **IZCM-1** (0.89 g, yield 81.0%): m.p. 232-234 °C. ^{1}H NMR (400 MHz, methanol-d_4) δ 8.34 (s, 1H), 7.44 -7.31 (m, 3H), 7.18 (d, J = 8.6 Hz, 2H), 6.94 (t, J = 8.8 Hz, 2H), 6.82 (d, J = 8.8 Hz, 2H), 6.65 (dd, J = 9.0, 2.4 Hz, 1H), 6.43 (d, J = 2.3 Hz, 1H), 3.38 (q, J = 7.0 Hz, 4H), 3.12 (t, J = 5.0 Hz, 4H), 2.51 (t, J = 5.0 Hz, 4H), 2.25 (s, 3H), 1.12 (t, J = 7.1 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 162.0, 161.9, 156.0, 151.0, 150.5, 141.4, 138.8, 136.4, 131.5, 129.7, 129.4, 129.3, 128.6, 127.0, 115.7, 115.1, 109.8, 108.9, 108.9, 97.0, 55.0, 48.5, 46.1, 44.9, 12.5. Purity: 99.6% by HPLC. HRMS (ESI) m/z: calcd for $C_{33}H_{34}N_5O_2F$: 552.2775 $[M+H]^+$, found 552.2765 $[M+H]^+$.



Rank	Score	Formula (M)	Ion	Meas. m/z	Pred. m/z	Df. (mDa)	Df. (ppm)	Iso	DBE
3	73.82	C33 H34 N5 O2 F	[M+H] +	552.2765	552.2775	-1.0	-1.81	75.35	19.0

Figure S1. HRMS spectrum of **IZCM-1**.

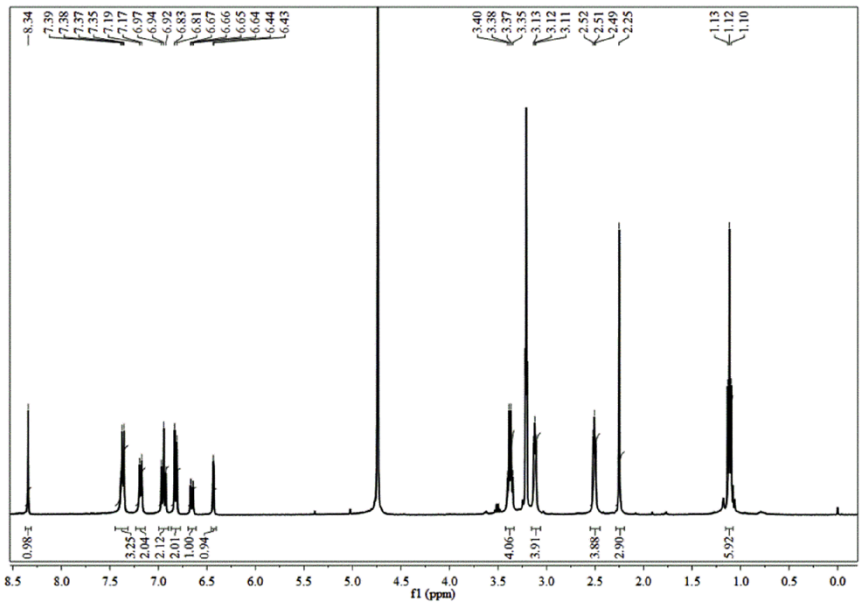


Figure S2. ^{1}H NMR spectrum of **IZCM-1**.

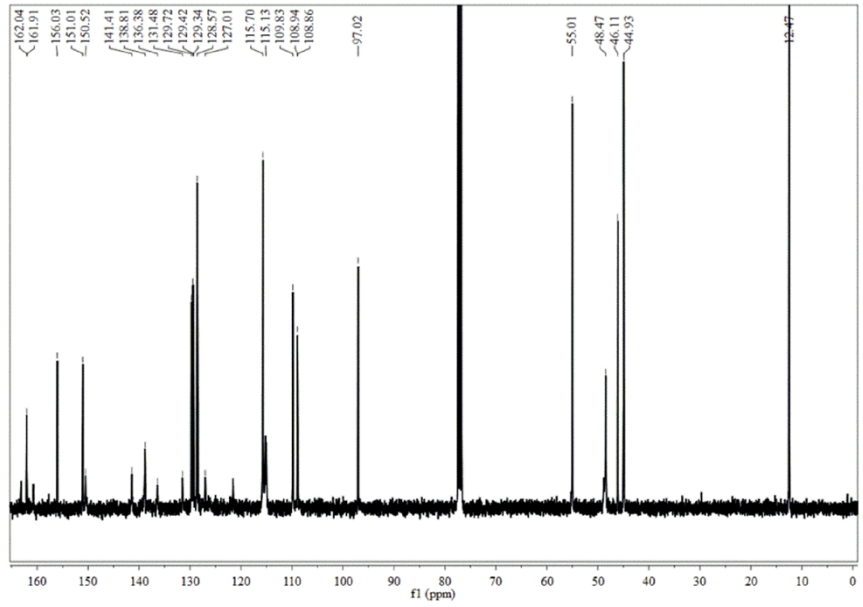


Figure S3. ^{13}C NMR spectrum of **IZCM-1**.

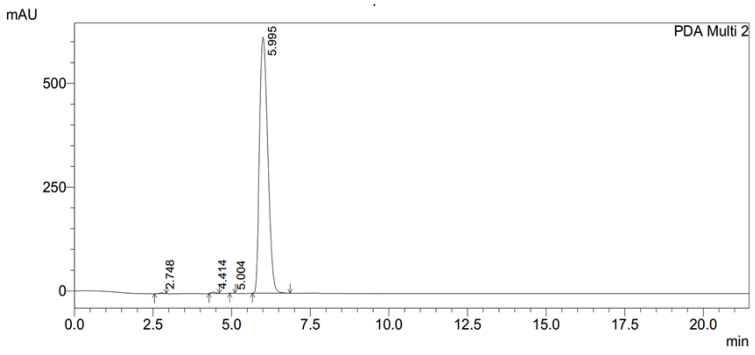


Figure S4. HPLC analysis of **IZCM-1**.

1.2 Materials

Table S1. DNA samples used in the present study.

Name	Sequence	Structure in K^+ Solution
Kras	5′-d(AGGGCGGTGTGGGAAGAGGGAAGAGGGGGAGG)-3′	Parallel G4
Pu22	5′-d(TGAGGGTGGGTAGGGTGGGTAA)-3′	Parallel G4
bcl-2	5′-d(GGGCGGGCGCGGGAGGAAGGGGGCGGG)-3′	Parallel G4
c-kit2	5′-d(GGGCGGGCGCGAGGGAGGGG)-3′	Parallel G4
Hras	5′-d(TCGGGTTGCGGGCGCAGGGCACGGGCG)-3′	Antiparallel G4
G3T3	5′-d(GGGTTTGGGTTTGGGTTTGGG)-3′	Antiparallel G4
TBA	5′-d(GGTTGGTGTGGTTGG)-3′	Antiparallel G4
htg22	5′-d(AGGGTTAGGGTTAGGGTTAGGG)-3′	Hybrid-Type G4
UspBQ1	5′-d(CAGGGTTAAGGGTATAACTTTAGGGGTTAGGGTT)-3′	Hybrid-Type G4
UspBQ2	5′-d(TAGGGTTAAGGGTATAACGTTAAGGGTTAGGGTT)-3′	Hybrid-Type G4
ds26	5′-d(CAATCGGATCGAATTCGATCCGATTG)-3′	Duplex
hairpin	5′-d(CGCGCGCGTTTTCGCGCGCG)-3′	Duplex
ctDNA	Calf Thymus DNA	Duplex
mutPu22	5′-d(TGAGCGTGGCGAGCGTGGCGAA)-3′	Single-Strand
Py22	5′-d(TTACCCACCCTACCCACCCTCA)-3′	Single-Strand
T21	5′-d(TTTTTTTTTTTTTTTTTTTT)-3′	Single-Strand
A21	5′-d(AAAAAAAAAAAAAAAAAAAA)-3′	Single-Strand
TAA	T21/$(A21)_2$	Triplex
htg22-Ap1	5′-d(ApGGGTTAGGGTTAGGGTTAGGG)-3′	Hybrid-Type G4
htg22-Ap7	5′-d(AGGGTTApGGGTTAGGGTTAGGG)-3′	Hybrid-Type G4
htg22-Ap13	5′-d(AGGGTTAGGGTTApGGGTTAGGG)-3′	Hybrid-Type G4
htg22-Ap19	5′-d(AGGGTTAGGGTTAGGGTTApGGG)-3′	Hybrid-Type G4
Pu22-Ap3	5′-d(TGApGGGTGGGGAGGGTGGGGAA)-3′	Parallel G4
Pu22-Ap7	5′-d(TGAGGGApGGGGAGGGTGGGGAA)-3′	Parallel G4
Pu22-Ap12	5′-d(TGAGGGTGGGGApGGGTGGGGAA)-3′	Parallel G4
Pu22-Ap16	5′-d(TGAGGGTGGGGAGGGApGGGGAA)-3′	Parallel G4
SPR- HTG	5′-biotin-d(GTTAGGGTTAGGGTTAGGGTTAGGGTTAGG)-3′	Hybrid-Type G4
SPR-c-MYC	5′-biotin-d(ACGTACGTGGGGAGGGTGGGGAGGGTGGGGAAGGTGGGG)-3′	Parallel G4
SPR-Duplex	5′-biotin-d(TTCGCGCGCGTTTTCGCGCGCG)-3′	Duplex

All oligonucleotides used in this study were purchased from Invitrogen (China) and Sangon (China). 7-diethylamino-4-methylcoumarin, calf thymus DNA (ctDNA), PEG 200 and Ficoll 70 were purchased from Sigma-Aldrich (Singapore). GelRed was purchased from Biotium (USA). All the oligonucleotides and ctDNA were dissolved in relevant buffer. Their concentrations were determined from the absorbance at 260 nm, respectively on the basis of respective molar extinction coefficients using NanoDrop 1000 Spectrophotometer

(Thermo Scientific, USA). To obtain G-quadruplex formation, oligonucleotides were annealed in relevant buffer containing KCl by heating to 95 °C for 5 min, followed by gradual cooling to room temperature. The oligonucleotides were engaged in G-quadruplex formation, as determined by circular dichroism (CD) measurements (**Figure S8**). Stock solutions of **IZCM-1** (10 mM) were dissolved in DMSO and stored at -80 °C. Further dilutions of samples and **IZCM-1** to working concentrations were made with relevant buffer immediately prior to use.

1.3 UV-Vis Spectroscopic Studies

UV-Vis spectroscopic studies were performed on a UV-2450 spectrophotometer (Shimadzu, Japan) using 1 cm path length quartz cuvette. For titration experiment, all oligonucleotides were firstly prepared through heating at 95 °C for 5 min followed with slow cooling to room temperature. Small aliquots of a stock solution of oligonucleotide were added into the solution containing **IZCM-1** at fixed concentration (5 μM) in Tris-HCl buffer (10 mM, pH 7.2) with 100 mM KCl. The final concentration of oligonucleotide was varied from 0 to 7.5 μM. After each addition of oligonucleotide, the reaction was stirred and allowed to equilibrate for at least 2 min and absorbance measurement was taken. The binding affinities of **IZCM-1** for G-quadruplexes were determined by fitting the absorption change at 445 nm to the Scatchard binding model.[3]

1.4 Fluorescence Studies

Fluorescence studies were performed on a LS-55 luminescence spectrophotometer (Perkin-Elmer, USA). A quartz cuvette with 2 mm × 10 mm path length was used for the spectra recorded at 5 nm excitation and emission slit widths unless otherwise specified.

The fluorescence quantum yield (Φ_F) of **IZCM-1** was calculated relative to a standard solution of rhodamine 123 in ethanol (Φ_F = 0.90) and was determined using the following formula: $\Phi_u = \Phi_s (A_u/A_s) \times (I_u/I_s)$, where Φ is the fluorescence quantum yield, I is the measured integrated emission intensity, and A is the optical density (absorbance). The u refers to the compound (**IZCM-1**) of unknown quantum yield, and s refers to the reference compound (Rohdamine 123) of known quantum yield. The fluorescence quantum yield (Φ_F) of 7-diethylamino-4-methylcoumarin was calculated relative to its previous reported fluorescence quantum yield in ethanol (Φ_F = 0.73) and was determined using above formula. The fluorescence spectra were recorded at 5 nm excitation and emission slit widths for the determination of Φ.

For titration experiment, all oligonucleotides were firstly prepared through heating at 95 °C for 5 min followed with slow cooling to room temperature. Small aliquots of a stock solution of sample (oligonucleotides,

ctDNA) were added into the solution containing **IZCM-1** at fixed concentration (1 μM) in Tris-HCl buffer (10 mM, pH 7.2) with 100 mM KCl. The final concentration of sample was varied from 0 to 10 μM. After each addition of sample, the reaction was stirred and allowed to equilibrate for at least 1 min and fluorescence measurement was taken at Ex 450 nm.

For 2-Ap titration experiment, **IZCM-1** were added into the solution containing Ap-labeled oligonucleotides at fixed concentration (300 nM) in Tris-HCl buffer (10 mM, pH 7.2) with 100 mM KCl. The final concentration of **IZCM-1** was varied from 0 to 3 μM. After each addition of **IZCM-1**, the reaction was stirred and allowed to equilibrate for at least 1 min and fluorescence measurement was taken at Ex 305 nm.

1.5 CD Studies

CD studies were performed on a Chirascan circular dichroism spectrophotometer (Applied Photophysics, UK). A quartz cuvette with a 4 mm path length was used for the recording of spectra over a wavelength range of 230–330 nm with a 1 nm bandwidth, 1 nm step size and time of 0.5 s per point. CD melting was performed at a fixed concentration of G-quadruplex (3 μM), either with or without a fixed concentration (15 μM) of **IZCM-1** in Tris-HCl buffer (10 mM, pH 7.2) with 100 mM KCl. The data was recorded at intervals of 2.5 °C over a range of 25–95 °C, with a heating rate of 0.5 °C/min. A buffer baseline was collected in the same cuvette and was subtracted from the sample spectra. Final analysis of the data was conducted using Origin 7.0 (OriginLab Corp.).

1.6 SPR Studies

SPR measurements were performed on a ProteOn XPR36 Protein Interaction Array system (Bio-Rad Laboratories, CA) using a Streptavidin-coated GLH sensor chip. Biotinylated oligonucleotides (SPR-HTG, SPR-c-MYC, SPR-Duplex) were attached to the chip. In a typical experiment, biotinylated DNA was folded in filtered and degassed running buffer (50 mM Tris-HCl, 100 mM KCl, pH 7.4). The DNA samples were then captured (~1000 RU) in five flow cell, leaving one flow cell as a blank. Solutions of **IZCM-1** were prepared with running buffer through serial dilutions of stock solution. Five concentrations were injected simultaneously at a flow rate of 50 μL/min for 400 s of association phase, followed with 400 s of dissociation phase at 25 °C. The GLH sensor chip was regenerated with short injection of 50 mM NaOH between consecutive measurements. The final graphs were obtained by subtracting blank sensorgrams from different DNA sensorgrams. Data were analyzed with ProteOn manager software, using the Langmuir model for fitting kinetic data.

1.7 Molecular Modeling Studies[4]

The G-quadruplex structures of Pu22 and htg22 were generated based on their NMR structures (PDB ID: 2L7V and 2JPZ) using Discovery Studio 2.5 and optimized with AMBER10.[5] Structure of IZCM-1 was constructed and optimized with Gaussian 03 using the HF/6-31G* basis set.[6] Docking analyses were performed with the AUTODOCK 4.2 program.[7] The dimensions of the active site box that was placed at the center of the G-quadruplex were set to 110 Å × 110 Å × 110 Å, with a grid spacing of 0.375 Å. Two hundred independent docking runs were performed for each model using the Lamarckian genetic algorithm (LGA). Simulations of molecular dynamics (MD) were performed using the sander module of the AMBER 10.0 program suite. The studied nucleic acids were treated using the parm99bsc0 parameters.[8] Partial atomic charges for IZCM-1 were derived using the HF/6-31G* basis set, followed by an RESP calculation, while force-field parameters were obtained from the generalized Amber forcefield (GAFF) using the ANTECHAMBER module. The K^+ radius was maintained at 2.025 Å.[4] Periodic boundary conditions were applied using the particle-mesh Ewald (PME) method, which was employed to treat long-range electrostatic interactions. The complexes of G-quadruplex and IZCM-1 were solvated in a rectangular box of TIP3P water molecules with solvent layers of 10 Å. Potassium counterions were added to neutralize the complexes. The hydrogen bonds were constrained using SHAKE. For the nonbonded interactions, a residue-based cutoff of 10 Å was applied. Temperature regulation was achieved via Langevin coupling with a collision frequency of 1.0. The solvated structures were subjected to initial minimization to equilibrate the solvent and counter cations. The G-quadruplex and inner K^+ ions were initially fixed with force constants of 100 kcal/mol. The system was then heated from 0 to 300 K in a 100 ps simulation, followed by a 100 ps simulation to equilibrate the density of the system. Subsequently, a constant pressure MD simulation of 10 ns was performed in an NPT ensemble at 1 atm and 300 K. The output and trajectory files were saved every 0.1 and 1 ps for subsequent analysis, respectively. The complete trajectory analysis was performed with the Ptraj module in the Amber 10.0 suite. The molecular mechanics Poisson-Boltzmann surface area (MM/PBSA) method implemented in the AMBER 10 suite was applied to calculate the binding free energy between the G-quadruplexes and the IZCM-1. All of the water molecules and counter ions were stripped off, but the K^+ present within the negatively charged central channel was included. A set of 1000 snapshots from the MD trajectories was collected to calculate the binding free energies. The average structure from the last 2ns was visualized using the PyMOL software package.

1.8 Gel Electrophoresis Studies

Different oligonucleotides were loaded onto a 20% bisacrylamide gel in 1×TBE buffer containing 100 mM KCl and were electrophoresed at 4 °C. Oligonucleotides were stained by **IZCM-1** (4 μg/mL, 20 mins), or by conventional staining agent GelRed (4 μg/mL, 20 mins).[9] DNA fragments were visualized under UV light and photographed using AlphaImager EC (ProteinSimple).

2. Other Supporting Table, Spectra and Graphs

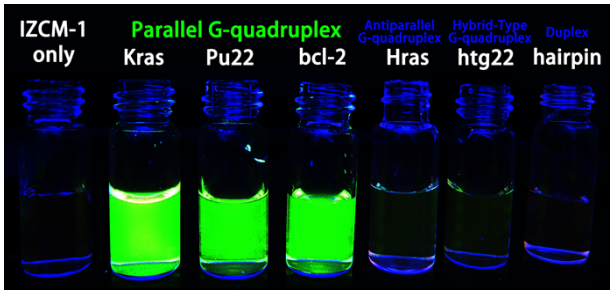


Figure S5. Photograph of 1 μM **IZCM-1** with and without 5 μM DNA samples in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2, taken under UV light (λ_{ex} = 302 nm).

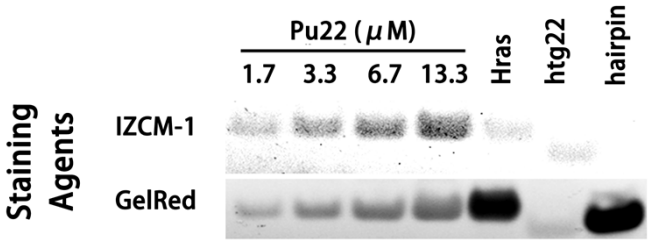


Figure S6. Electrophoresis staining of DNA samples by 4 μg/mL **IZCM-1** or GelRed.

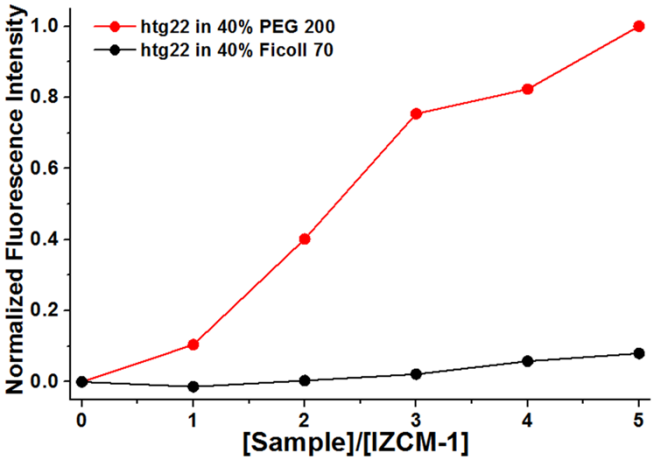


Figure S7. Fluorescence enhancement of **IZCM-1** with stepwise addition of G-quadruplex htg22 in different

crowding solution.

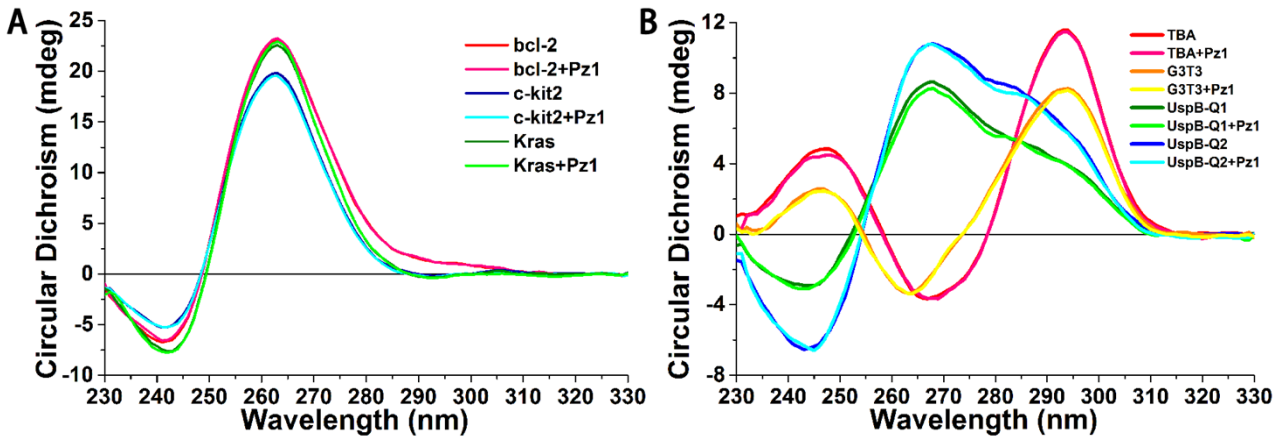


Figure S8. CD spectra of 3 μM G-quadruplex samples in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2, with and without 15 μM **IZCM-1**. (A) Parallel G-quadruplexes. (B) Antiparallel and hybrid-type G-quadruplexes.

Table S2. Melting temperature (T_m, °C) of 3 μM G-quadruplex samples with and without 15 μM **IZCM-1** in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2.

	T_m without **IZCM-1**	T_m with **IZCM-1**	ΔT_m
Pu22	86.6 ± 0.6	90.1 ± 1.5	3.5 ± 2.0
htg22	67.5 ±0.2	66.4 ± 0.4	-1.1 ± 0.5
Hras	61.8 ±0.4	62.5 ± 0.4	0.7 ± 0.8

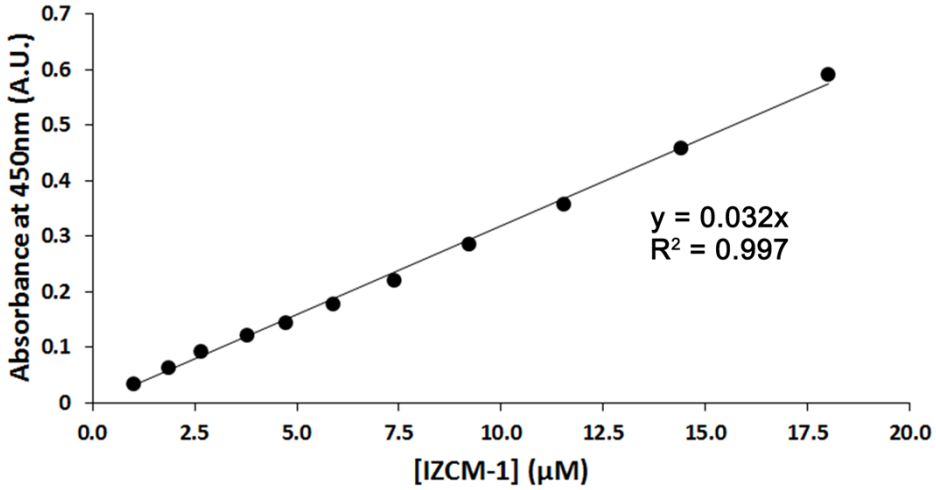


Figure S9. Concentration-Dependent UV-Vis absorbance of **IZCM-1**. All of the spectra were collected in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2. Concentration dependant UV-Vis absorbance spectra of **IZCM-1** showed that the variations follow the Beer-Lambert law.

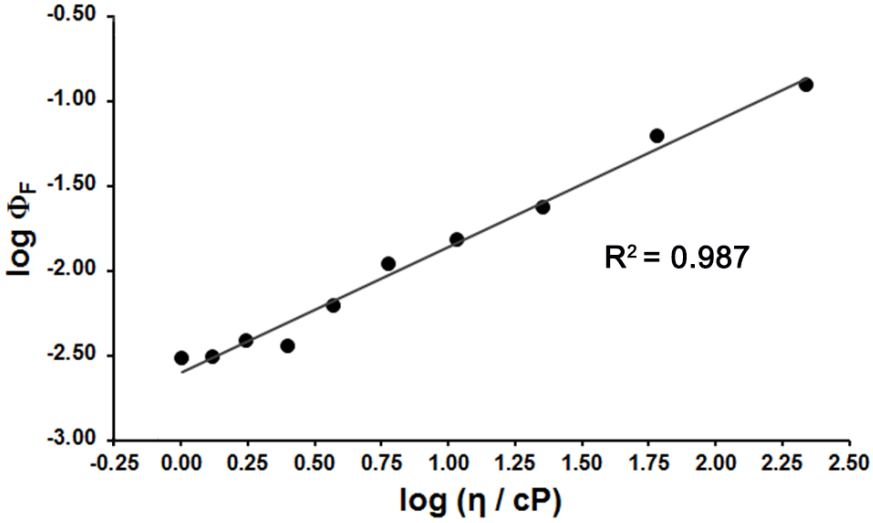


Figure S10. Viscosity dependence of the fluorescence quantum yield for **IZCM-1** in glycerol-water mixed solution.

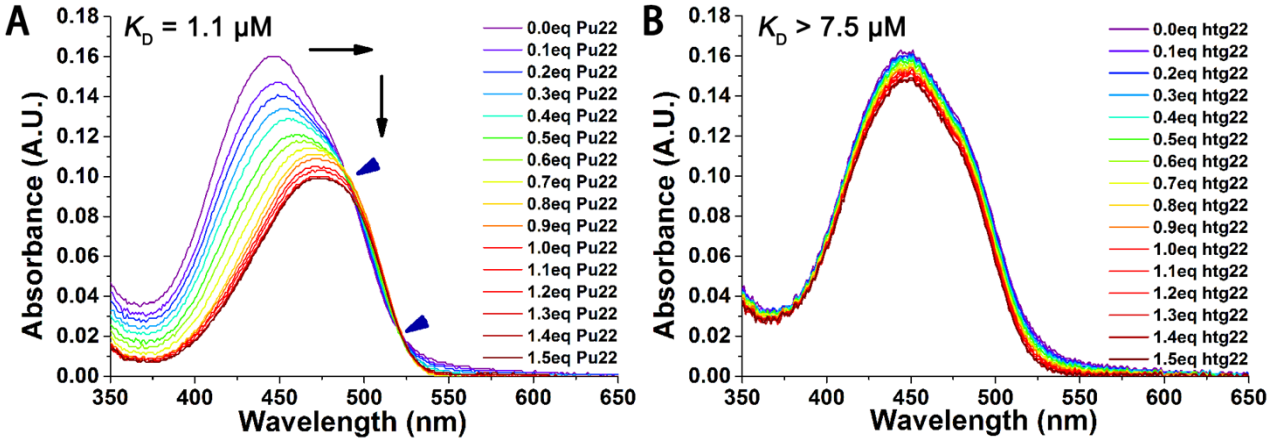


Figure S11. UV titrations of 5 μM **IZCM-1** with stepwise addition of G-quadruplex Pu22 (A) and htg22 (B) in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2.

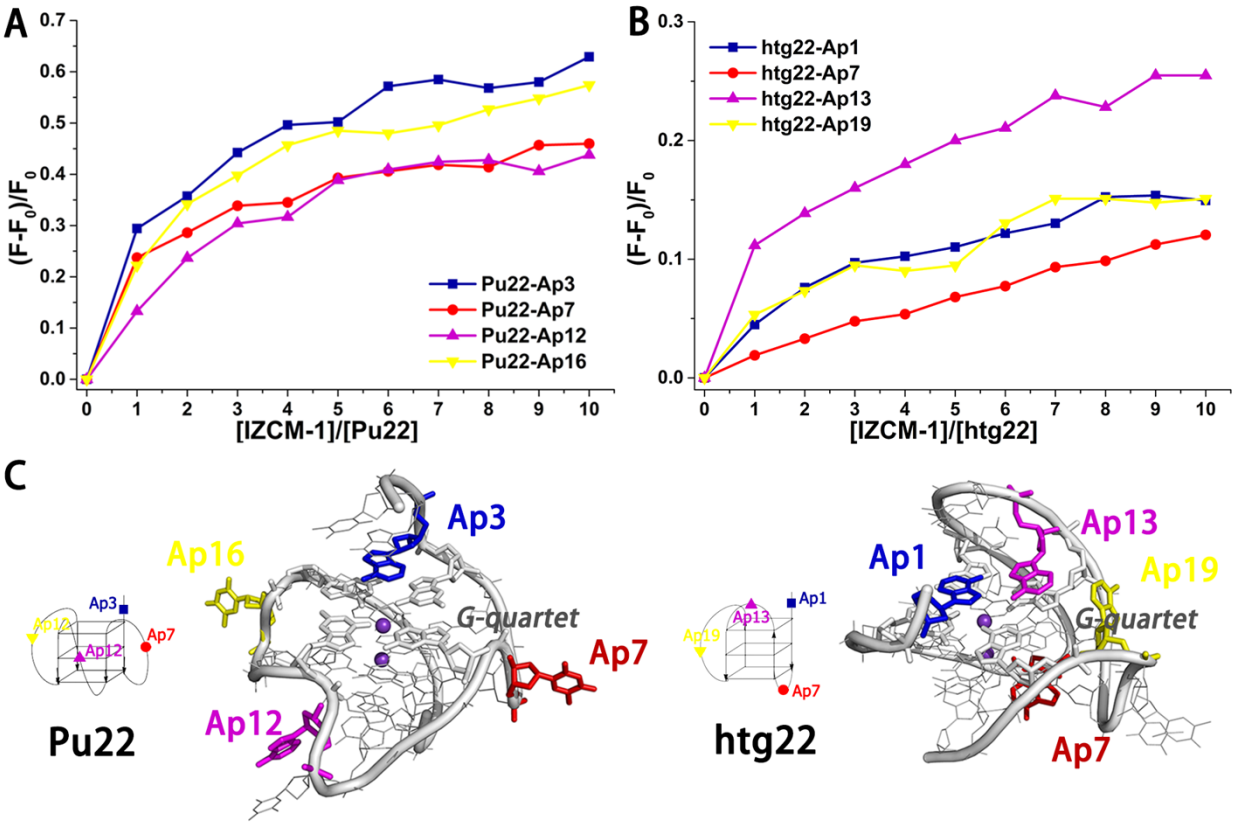


Figure S12. Fluorescence titrations of 300 nM 2-AP labeled G-quadruplexes with stepwise addition of **IZCM-1** in 10 mM Tris-HCl buffer, 100 mM KCl, pH 7.2. (A) Plot of normalized fluorescence intensity at 375 nm of 2-Ap individually labeled Pu22 versus binding ratio of [**IZCM-1**]/[Pu22]. (B) Plot of normalized fluorescence intensity at 375 nm of 2-Ap individually labeled htg22 versus binding ratio of [**IZCM-1**]/[htg22]. (C) Illustration of the 2-Ap labeled sites in Pu22 correlated to the binding model of **IZCM-1** to Pu22 in Fig. 4. (D) Illustration of the 2-Ap labeled sites in htg22 correlated to the binding model of **IZCM-1** to htg22 in Fig. 4.

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