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20. Paper Based Assay

1] Citrate-capped gold nanoparticle synthesis (Turkevich method)

Overview / principle: $HAuCl_4$ (tetrachloroauric acid) is reduced by trisodium citrate in boiling water; citrate acts as both reducing agent and capping (stabilizing) ligand.

Reagents & materials

- Chloroauric acid solution $HAuCl_4 \cdot 3H_2O$ (solid) or a standard commercial aqueous solution (prepare fresh).
- Trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$) — analytical grade.
- Ultrapure water (Milli-Q or equivalent).
- Glassware: 250 mL round-bottom flask or 250 mL borosilicate beaker, reflux condenser optional.
- Hotplate with magnetic stirrer, stir bar.
- Thermometer.
- Volumetric flasks/pipettes, graduated cylinders.
- pH paper (optional).
- UV-Vis spectrophotometer (for characterization).
- Centrifuge (optional for concentration/washing).
- Clean glass vials for storage (amber if possible).

Recipe (final volume 100.0 mL)

Target concentrations/formulation 100 mL batch (~20 nm)

- **Gold (Au^{3+}): 0.25 mM final** in 100 mL.
    - Moles Au = 0.25 mM × 0.100 L = 2.50×10^{-5} mol
    - Mass $HAuCl_4\cdot3H_2O$ required ≈ **8.50 mg** (MW ≈ 339.79 $g\cdot mol^{-1}$).
- **Trisodium citrate (1% w/v stock)** — prepare 1 g per 100 mL (10 $g\cdot L^{-1}$; ≈ 34.0 mM).
    - **Add 1.0 mL** of this 1% citrate solution to the boiling gold solution (final citrate:Au molar ratio ≈ 1.36).

If you have a commercial $HAuCl_4$ solution, calculate the volume needed to give 2.50×10^{-5} mol Au (use the supplier concentration).

Preparation of stock solutions

1. **One% (w/v) trisodium citrate stock** (fresh):
    - Weigh **1.00 g** trisodium citrate dihydrate and dissolve in **100.0 mL** ultrapure water. Store at room temp for short term (use fresh within days).
2. **$HAuCl_4$ solution** (if starting from solid):
    - Weigh **8.50 mg** $HAuCl_4\cdot3H_2O$ and dissolve in ~50 mL ultrapure water, dilute to **100.0 mL** to make a 0.25 mM solution (or directly dissolve the 8.50 mg into the 100 mL reaction volume; see procedure below). If using a concentrated commercial solution, calculate the required volume.

Step-by-step procedure

1. **Glassware prep / cleaning:** Wash glassware with laboratory detergent, rinse thoroughly with tap water then with ultrapure water. Use clean equipment — colloids are sensitive to contaminants.
2. **Set up:** Place 100 mL reaction vessel on hotplate with magnetic stir bar. Add **100.0 mL** ultrapure water (or dissolve the measured $HAuCl_4$ directly into the vessel — see step 3).

3. **Add gold salt:** Add **8.50 mg** $HAuCl_4 \cdot 3H_2O$ to the 100 mL water (gently stir until fully dissolved). If using a concentrated $HAuCl_4$ stock, add volume equivalent to 0.25 mM final (calculate accordingly).
4. **Heat to boiling:** Bring solution to a rolling boil under vigorous stirring. Use a reflux condenser or watch for evaporation; do not boil dry.
5. **Rapid addition of citrate:** Quickly add **1.0 mL** of the 1% trisodium citrate stock to the boiling gold solution **all at once** while stirring vigorously.
6. **Observe color change:** Within 30 s–2 min the solution will change color: pale → clear → purple → deep red wine (for ~20 nm). Continue boiling + stirring for **8–10 minutes** to complete the reaction.
7. **Cool:** Remove from heat and let cool to room temperature while stirring gently (~15–30 min). Transfer to a clean container and cover.
8. **Storage:** Store at 4 °C (fridge) in amber/high-quality glass for weeks to months. Avoid freezing and long exposure to light.

Expected results / characterization

- **Color:** deep wine-red (for ~20 nm AuNPs).
- **UV-Vis:** strong SPR peak around **~520–525 nm** (peak position and width depend on size & aggregation). Narrow peak ≈ monodisperse.
- **DLS / TEM:** if available, DLS should show hydrodynamic diameter around ~20–30 nm (including citrate layer); TEM shows core ~20 nm.

Tips & troubleshooting

- **If solution stays pale/yellow:** insufficient reduction — check citrate freshness and that solution was vigorously boiling when citrate was added.
- **If solution turns blue/gray or shows visible aggregates:** overgrowth/aggregation. Try lower citrate:gold ratio (or prepare fresh glassware and ultrapure water). Centrifugation + resuspension can remove aggregates.
- **Smaller particles (~10 nm):** increase citrate:Au ratio (e.g., use a higher volume or concentration of citrate). For smaller sizes you can start with higher citrate (e.g., 2–3 mL of 1% into 100 mL of 0.25 mM) — size tuning is empirical: more citrate → smaller average diameter.
- **Larger particles (>20 nm):** lower citrate:Au ratio, or seed-growth methods.

- **pH:** neutral to slightly acidic/basic is typical; strong pH changes can affect nucleation and stability.

Safety

- $HAuCl_4$ is hazardous (oxidizer, toxic by ingestion/inhalation); handle with gloves, eye protection, and in a fume hood.
- Trisodium citrate is low hazard but use PPE.
- Hot liquids — avoid burns; use proper heat-resistant gloves and caution.
- Dispose of Au-containing waste according to institutional hazardous metal waste procedures.

Two] Preparation of Graphene Oxide (GO) Suspension

Procedure

1. **Weighing:** Accurately weighed **1 mg of Graphene Oxide (GO)**.
2. **Dispersion:** Transferred GO into a clean beaker/vial containing **20 mL of Milli-Q water**.
    - This yields a **0.5 mg/mL GO suspension**.
3. **Sonication:** The suspension was sonicated at **40 Hz** for **30 minutes** at room temperature to ensure uniform dispersion.
4. **Spectral Analysis:**
    - The GO suspension was analyzed using a **UV–Vis spectrophotometer**.
    - Absorbance was recorded across the 200–800 nm range.
    - The suspension showed a characteristic absorption peak around **450 nm.**

3] Preparation of GO working concentrations (from 50 µg/mL stock)

Prepare 1 mL each of 50, 25, 12.5, 6.25 and 3.125 µg/mL graphene oxide (GO) in distilled water (d/w) for downstream assays.

- GO stock: **50 µg/mL** (aqueous; well suspended)
- Distilled water (d/w) or Milli-Q water
- Calibrated micropipettes and tips
- 1.5 mL low-bind microcentrifuge tubes (amber or foil-wrapped if light sensitivity is a concern)
- Vortex mixer and (optional) ultrasonic bath (40 kHz)

Table 2a: Various concentration of GO preparation.

Target Conc. (µg/mL)	Dilution Factor	Stock Volume (µL)	Diluent (d/w) Volume (µL)	Final Volume (µL)
50	1:1 (Stock)	1000	0	1000
25	1:2	500	500	1000

Target Conc. (µg/mL)	Dilution Factor	Stock Volume (µL)	Diluent (d/w) Volume (µL)	Final Volume (µL)
12.5	1:4	250	750	1000
6.25	1:8	125	875	1000
3.125	1:16	62.5	937.5	1000

4] FITC Stock & Dilutions

Stock solution (main stock, light-protected)

- **Compound:** FITC (Fluorescein Isothiocyanate)
- **MW:** 389 g/mol
- **Target stock: 1 mg/mL in DMSO**
- **Molarity check:**

  1 mg/mL=389 g/mol1×10−3 g/mL×1000 mL/L=2.57×10−3 mol/L=2.57 mM

How the DMSO stock was prepared

1. In low light (foil-wrapped bench) weigh **5 mg FITC** into an amber microcentrifuge tube.

2. Add **5 mL anhydrous DMSO** (molecular biology grade).
    - Result: **1 mg/mL (2.57 mM) FITC in DMSO**.
3. Mix: brief vortex (5–10 s) → gentle flick → spin down. If particulates remain, repeat; optionally **0.22 µm PTFE** filter (amber syringe).
4. **Aliquot** (e.g., 50–100 µL) into amber tubes, flush headspace with inert gas if available.
5. **Storage:** −20 °C (or −80 °C for long term), protected from light. Avoid >3 freeze–thaw cycles.
6. **Note:** DMSO is not prepared in the laboratory; fresh, dry (anhydrous) solvent is to be used in order to avoid hydrolysis and dye degradation. It should be handled with gloves, as it facilitates transdermal absorption.

Intermediate aqueous working stock: 10 µM FITC

Prepare in **water or PBS** (light-protected) immediately before use.

- From **2.57 mM** DMSO stock to **10 mL of 10 µM**:

  V1=C1C2V2=2570 µM10 µM×10 mL=0.03891 mL=38.91 µL
- **Pipette: 39 µL** of 2.57 mM stock + **9961 µL** water → mix gently, keep protected from light.

Serial dilutions from 10 µM to nM (final volume 2 mL each)

Formula: $V_1 = \frac{C_2 V_2}{C_1}$, with $C_1 = 10{,}000\ \text{nM}$, $V_2 = 2000\ \mu L$.

Table 3a: Various concentration of GO preparation.

Target conc. (nM)	Final Volume (µL)	Volume of 10 µM stock (µL)	Volume of Water (µL)
100	2000	20	1980

Target conc. (nM)	Final Volume (µL)	Volume of 10 µM stock (µL)	Volume of Water (µL)
80	2000	16	1984
60	2000	12	1988
40	2000	8	1992
20	2000	4	1996

Five] FRET Mechanism

- **Interaction & adsorption:** FITC molecules adsorb onto graphene oxide (GO) surfaces via π–π stacking and hydrophobic/ electrostatic interactions. Adsorption brings the fluorophore into proximity with GO.
- **Quenching mechanisms:** Once closely associated, fluorescence is reduced by one or more mechanisms:
  - **Förster Resonance Energy Transfer (FRET):** non-radiative energy transfer from excited FITC (donor) to GO (acceptor) if spectral overlap and distance (<10 nm) permit.
  - **Photoinduced electron transfer (PET):** electron transfer from the excited dye to GO, causing nonradiative relaxation.
  - **Static (complex) quenching:** formation of non-fluorescent FITC–GO complexes.

- **Net effect:** FITC fluorescence (Ex 495 / Em 530) is decreased when FITC is adsorbed to GO. The degree of quenching depends on FITC concentration, surface coverage of GO, incubation time, and buffer conditions (pH, ionic strength).
- **Interpretation:** A decrease in fluorescence compared to FITC-only controls indicates adsorption/interaction. In competition or sensor assays, displacement or conformational change that releases FITC from GO will restore fluorescence (signal-on).

(ON/OFF States)

- **OFF State (Quenched)**:
  When **FITC (donor fluorophore)** is adsorbed onto the surface of **graphene oxide (GO, acceptor)**, the close proximity (<10 nm) enables **Förster Resonance Energy Transfer (FRET)**. Instead of emitting green fluorescence, the excited-state energy of FITC is non-radiatively transferred to GO, which acts as a quencher. As a result, FITC fluorescence at 530 nm is **suppressed (OFF state)**.
- **ON State (Fluorescent)**:
  When FITC is **not bound to GO**, or if the interaction is disrupted (e.g., by competition, conformational change, or desorption), the distance between donor and acceptor increases beyond the FRET efficiency range. In this case, FITC retains its normal excitation/emission (Ex 495 nm / Em 530 nm), producing **bright fluorescence (ON state)**.

Six] GO–FITC Assay

Assay: GO quenching of FITC fluorescence (endpoint)
Plate reader: Victor Nivo (PerkinElmer) — excitation **495 nm**, emission **530 nm**

Reagents & stock concentrations

- **GO stock:** (50, 25, 12.5, 3.125 µg/mL).
- **FITC working stocks:** 20, 40, 60, 80, 100 nM (prepared in water; protected from light)
- **Assay buffer:** Milli-Q, keep consistent across wells

Example

Protocol (stepwise)

1. A black, flat-bottom 96-well plate was pre-labeled prior to the assay.
2. Into each test well, 50 µL of 3.125-50 µg/mL GO stock was pipetted. For the GO-only control, 50 µL of GO stock and 50 µL of buffer were added.
3. Subsequently, 50 µL of each FITC working stock (20–100 nM) was dispensed into the appropriate wells. For FITC-only controls, 50 µL of FITC stock and 50 µL of water were added. All solutions were handled in separate blocks to minimize cross-contamination.
4. The plate was then gently mixed by pipetting up and down once or by brief shaking, taking care to avoid bubbles. The plate was kept protected from light during and after mixing.
5. The samples were incubated at room temperature for 10–15 minutes to allow adsorption and equilibration.
6. Fluorescence was then measured on the Victor Nivo plate reader with the following settings: excitation wavelength 495 nm, emission wavelength 530 nm, top-read mode, endpoint mode, total well volume of 100 µL, and medium gain. Raw RFU values were recorded, and blanks were subtracted.
7. Finally, the data were exported and plotted to compare FITC-only wells with GO+FITC wells in order to quantify the degree of fluorescence quenching.

% Quenching=FFITC onlyFFITC only−FGO+FITC×100

$$\% \text{ Quenching} = \frac{F_{\text{FITC only}} - F_{\text{GO+FITC}}}{F_{\text{FITC only}}} \times 100$$

Key:

- **Blank = Water only**
- **PC = Positive Control (FITC only, no GO)**
- **Exp = Experimental wells (GO + FITC)**

1	2	3	4	5	6	7	8	9	10	11	12

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	PC (20 nM)	Exp (20+50)	Exp (20+25)	Exp (20+12.5)	Exp (20+3.125)	PC (40 nM)	Exp (40+50)	Exp (40+25)	Exp (40+12.5)	Exp (40+3.125)	Blank
B	Blank	PC (60 nM)	Exp (60+50)	Exp (60+25)	Exp (60+12.5)	Exp (60+3.125)	PC (80 nM)	Exp (80+50)	Exp (80+25)	Exp (80+12.5)	Exp (80+3.125)	Blank
C	Blank	PC (100 nM)	Exp (100+50)	Exp (100+25)	Exp (100+12.5)	Exp (100+3.125)	Blank	Blank	Blank	Blank	Blank	Blank
D–H	**(**empty / replicates / repeats as needed)											

Explanation:

- **Blanks (water only) are placed at edges for background subtraction.**
- **Positive controls (FITC only) are included at each FITC concentration (20–100 nM).**
- **Experimental wells (Exp) cover all combinations of 5 FITC × 4 GO (20 total).**
- **Remaining rows (D–H) can be used for replicates or other conditions.**

Notes & precautions

- Protect FITC-containing solutions and plate from light.
- Run FITC-only controls at the *final* concentrations (i.e., after mixing) to compute correct quenching percentages — alternatively measure FITC-alone wells prepared with equal volume + buffer.

- If signal is weak or inconsistent, sonicate GO stock briefly before use (40 kHz, short) to re-disperse. Use low-bind plates/tubes to reduce adsorption loss.
- Record plate reader gain and other settings for reproducibility.
- Dispose of GO and FITC waste per institutional procedures.

7] Preparation of BSA + PBS Working Stocks and Dilutions

Main Stock (BSA + PBS)

1. **Weighed 265 mg of BSA**.
2. **Dissolved in 40 mL of 1× PBS (100 mM)** to prepare the **main stock**.

Working Stock (WS)

3. Prepared a **20 µg/mL working stock (200 nM)** by diluting:
    - 30 µL of main stock (super stock)
    - 970 µL of PBS.

Preparation of Dilutions (500 µL each from WS)

4. From the WS, the following dilutions were made:

Table 6c: various PBS concentration.

Target Conc. (µg/mL)	Approx. Conc. (nM)	Volume of WS (µL)	Volume of PBS (µL)	Final Volume (µL)
1 µg/mL	37.5 nM	62.5	437.5	500
2 µg/mL	75 nM	125	375	500
10 µg/mL	150 nM	250	250	500
15 µg/mL	225 nM	375	125	500

For Microplate Assay

5. Each well was loaded as follows:
    - **50 µL of GO solution** (at the desired test concentration)
    - **50 µL of prepared BSA + PBS dilution** (or PBS alone for control).
    - Total well volume = **100 µL**.

8] Preparation of reduced graphene oxide (rGO)

Materials

- rGO powder (weighed)
- Milli-Q water (or buffer if required)
- Ultrasonic water bath (≈40 kHz)
- Vortex mixer
- Calibrated micropipettes and low-bind tips/tubes
- Amber vials or aluminium foil (light protection optional)
- Microcentrifuge

Procedure

1. The rGO powder was accurately weighed (mass recorded).
2. The powder was transferred into a clean vial and Milli-Q water was added to reach the desired stock concentration (example below). The vial was capped.
3. The dispersion was sonicated in an ultrasonic bath (≈40 kHz) for 10–15 minutes at room temperature to obtain a homogeneous stock. Brief vortexing was used before and after sonication.
4. The stock was inspected visually; if large visible aggregates were present, the sample was centrifuged briefly (e.g., 3,000–5,000 × g, 5 min) and the supernatant collected.

5. Working dilutions were prepared from the sonicated stock by pipetting appropriate volumes into low-bind tubes and bringing to final volume with Milli-Q water (see dilution table). Tubes were gently vortexed to mix.
6. For microplate assays, intermediate dilutions were prepared to avoid pipetting <5 µL. Final well mixtures were prepared immediately before reading.
7. Stock and working solutions were stored at 4 °C (short term) and re-sonicated briefly prior to use if settling occurred.

Stock preparation :

1 mg/mL (1000 µg/mL) stock — 5 mg rGO powder was added to 5.0 mL Milli-Q water, capped, vortexed, and sonicated 10–15 min in an ultrasonic bath (≈40 kHz) to produce a well-dispersed 1 mg/mL stock.

Direct addition from the 1 mg/mL rGO stock to 100 µL wells was avoided for low concentrations, since volumes <5 µL were required. Instead, intermediate dilutions were prepared and used for plating in order to reduce pipetting error.

- To obtain a final concentration of 25 µg/mL in a 100 µL well, an intermediate 250 µg/mL solution was first prepared (250 µL of 1 mg/mL stock + 750 µL Milli-Q water → 1 mL). From this intermediate, 10 µL was added to the well along with 90 µL buffer, giving the desired final concentration.
- For 100 µg/mL final concentration, 10 µL of 1 mg/mL stock was added to the well along with 90 µL buffer.
- For 50 µg/mL final concentration, 5 µL of 1 mg/mL stock + 95 µL buffer would have been required, but to avoid pipetting <5 µL, an intermediate 500 µg/mL solution was prepared (500 µL stock + 500 µL water). From this intermediate, 10 µL was added to the well with 90 µL buffer, giving 50 µg/mL final.
- For 25 µg/mL final concentration, the 250 µg/mL intermediate described above was used. 10 µL of intermediate + 90 µL buffer was pipetted per well.
- For 10 µg/mL final concentration, an intermediate 100 µg/mL solution was prepared (100 µL stock + 900 µL water). From this, 10 µL was added to the well along with 90 µL buffer, giving 10 µg/mL final.

Table 8a: Different concentration of rGO.

Target conc (µg/mL)	Volume of 1 mg/mL stock (µL)	Volume Milli-Q (µL)	Dilution factor

Target conc (µg/mL)	Volume of 1 mg/mL stock (µL)	Volume Milli-Q (µL)	Dilution factor
1000 (stock)	1000	0	1:0
500	500	500	1:2
200	200	800	1:5
100	100	900	1:10
50	50	950	1:20
25	25	975	1:40
10	10	990	1:100

Nine] Assay Protocol for rGO–FITC Quenching

1. Preparation of Stock Solutions

- rGO powder was weighed and dissolved in Milli-Q water to prepare a stock solution of **1 mg/mL**. The suspension was sonicated for **10–15 minutes** to achieve uniform dispersion.
- FITC stock: **5 mg in 5 mL** (≈2.56 mM).
- A working stock of **1 mM FITC** was prepared by diluting **1.56 µL of FITC stock** in **3998 µL water**.
- Working concentrations of FITC (20, 40, 60, 80, 100 nM) were then prepared from the 1 mM stock using appropriate dilutions (e.g., 40 µL + 1960 µL water for 20 nM).

Assay Procedure

1. 50 µL of rGO solution (different dilutions) was pipetted into designated wells. For FITC-only control, 50 µL buffer was added.
2. 50 µL of FITC solution (at required concentrations) was added into respective wells.
3. The plate was gently mixed by pipetting up and down once, avoiding bubbles.
4. The plate was protected from light during incubation.
5. Incubation was carried out at room temperature for 15 minutes to allow adsorption and equilibration.

4. Fluorescence Measurement

- The plate was read using Victor Nivo multimode plate reader.
- Excitation: 495 nm, Emission: 530 nm.
- Mode: Top read, endpoint, 100 µL well volume, medium gain.
- Raw RFU values were recorded.
- Blank-subtracted values were used for data analysis.

5. Data Analysis

- FITC-alone wells were used as reference (100% fluorescence).
- Fluorescence from FITC + rGO wells was compared to calculate % quenching.

Table 9a: FITC Only (20–100 nM)

Column	Sample	Description
1	20 nM FITC	FITC only
2	40 nM FITC	FITC only
3	60 nM FITC	FITC only
4	80 nM FITC	FITC only
5	100 nM FITC	FITC only
6	20 nM FITC (PC)	Positive control
14	Blank	Milli-Q water

Table 9b: rGO with Constant 20 nM FITC

Column	rGO Conc.	FITC Conc.	Description
7	1 mg/mL	20 nM	Highest rGO, constant FITC
8	0.5 mg/mL	20 nM	Half conc. rGO + FITC
9	0.2 mg/mL	20 nM	rGO + FITC
10	0.1 mg/mL	20 nM	rGO + FITC
11	0.05 mg/mL	20 nM	rGO + FITC
12	0.025 mg/mL	20 nM	rGO + FITC
13	0.01 mg/mL	20 nM	Lowest rGO, constant FITC

Table 9c: Replicates

- Rows A, B, C: All conditions above are repeated in triplicate.
- Each well volume: 100 µL (50 µL rGO + 50 µL FITC).

Col	1	2	3	4	5	6	7	8	9	10	11	12	13	14
A	20 nM FITC	40 nM FITC	60 nM FITC	80 nM FITC	100 nM FITC	PC (20 nM FITC)	rGO 1 mg/mL + 20 nM FITC	rGO 0.5 mg/mL + 20 nM FITC	rGO 0.2 mg/mL + 20 nM FITC	rGO 0.1 mg/mL + 20 nM FITC	rGO 0.05 mg/mL + 20 nM FITC	rGO 0.025 mg/mL + 20 nM FITC	rGO 0.01 mg/mL + 20 nM FITC	Blank (Buffer)
B	20 nM FITC	40 nM FITC	60 nM FITC	80 nM FITC	100 nM FITC	PC (20 nM FITC)	rGO 1 mg/mL + 20 nM FITC	rGO 0.5 mg/mL + 20 nM FITC	rGO 0.2 mg/mL + 20 nM FITC	rGO 0.1 mg/mL + 20 nM FITC	rGO 0.05 mg/mL + 20 nM FITC	rGO 0.025 mg/mL + 20 nM FITC	rGO 0.01 mg/mL + 20 nM FITC	Blank (Buffer)
C	20 nM FITC	40 nM FITC	60 nM FITC	80 nM FITC	100 nM FITC	PC (20 nM FITC)	rGO 1 mg/mL + 20 nM FITC	rGO 0.5 mg/mL + 20 nM FITC	rGO 0.2 mg/mL + 20 nM FITC	rGO 0.1 mg/mL + 20 nM FITC	rGO 0.05 mg/mL + 20 nM FITC	rGO 0.025 mg/mL + 20 nM FITC	rGO 0.01 mg/mL + 20 nM FITC	Blank (Buffer)

- Each well = **100 µL** total (50 µL rGO + 50 µL FITC).
- Columns **1–5** = FITC only controls.
- Column **6** = FITC positive control (20 nM).
- Columns **7–13** = rGO quenching with constant FITC (20 nM).
- Column **14** = Blank (Milli-Q water or buffer).
- Rows **A–C** = technical triplicates.

10] Aptamer Stocks Preparation

Materials: FAM-labelled aptamer (lyophilized), non-labelled aptamer, TE buffer (0.1 M, 1×), nuclease-free water (NFW), foil (to protect FAM), calibrated pipettes, low-bind tubes.

What was done

100 µM aptamer stocks (both FAM-labelled and non-labelled) were prepared by dissolving lyophilized aptamer in TE buffer (10 mM Tris, 1 mM EDTA, pH ~7.5), in accordance with vendor instructions. The solutions were mixed briefly by vortexing and briefly centrifuged to collect contents at the bottom of the tube. Aliquots were stored at −20 °C to avoid repeated freeze–thaw events. The FAM-labelled aptamer tubes were protected from light by wrapping in foil

1. The 1× TE buffer (0.1 M) was used as the working buffer.
2. **Sequence 1 (non-FAM)**: the aptamer vial (100 µM) was reconstituted by adding 761 µL TE into the vial. The tube was mixed and stored (conditions recorded).
3. **Sequence 2 (FAM-labelled)**: the aptamer vial was reconstituted by adding 956 µL TE; the tube was covered in foil to protect from light. The tube was mixed and stored.
4. The reconstituted aptamers were vortexed 1 min and stored at −20 °C (light-protected for FAM).

Preparation of working dilutions of FAM-labelled aptamer (from 100 µM stock)

Formula used ($C_1V_1 = C_2V_2$). Note: 100 µM = **100,000 nM**.

To make 2 mL final:

10 nM final: $V1=C_2V_2/C_1$

=10 nM×2000 µL/100000 nM

= 0.2 µL of 100 µM

= 1999.8 µL NFW was added as the makeup amount

20 nM final: 0.4 µL of 100 µM + 1999.6 µL NFW.

***Safety Considerations**

All FAM-labelled aptamer preparations were protected from light during handling and storage to prevent photobleaching, and were stored at −20 °C in opaque or foil-wrapped tubes.

11]Fentanyl stock & preparation

Main fentanyl stock (in methanol) was present at **50 ng/mL** which corresponds to **148.8 µM**. This was treated as the working main stock (in methanol).

1. The main fentanyl stock solution (148.8 µM in methanol, 50 ng/µL) was thawed and equilibrated in the hood.
2. Pre-labelled sterile polypropylene tubes were placed inside the hood for each working concentration (5, 10, 15, 20 nM).
3. Using a micropipette, the required volume of fentanyl stock (see dilution table) was carefully pipetted into each tube.
4. Milli-Q water (diluent) was added to bring the total volume to 10 mL.
5. Each solution was mixed gently by pipetting up and down, vortexing was avoided to prevent aerosol formation.
6. The prepared working solutions were transferred to amber cryovials (to protect from light) and stored at –20 °C until use.
7. The hood surface was cleaned with 70% ethanol followed by 10% bleach after completion.

Calculation example ($C_1V_1 = C_2V_2$), using C_1 = 148.8 µM = 148,800 nM.

Table 11a: Fentanyl from 148.8 µM stock final volume 10ml

Target Conc. (nM)	Stock Volume (µL)	Water Volume(µL)
5 nM	0.34 µL	9,999.66 µL
10 nM	0.67 µL	9,999.33 µL
15 nM	1.01 µL	9,998.99 µL

Target Conc. (nM)	Stock Volume (µL)	Water Volume(µL)
20 nM	1.34 µL	9,998.66 µL

Safety Precautions

- All steps were performed inside a certified fume hood.
- PPE: double gloves, lab coat, goggles, respirator/N95.
- Used polypropylene tubes and filtered tips.
- Waste was collected in dedicated opioid containers.
- Hood surface was decontaminated with 70% ethanol + 10% bleach after use.

12] UV–Visible Spectrophotometric Analysis

Samples Analyzed

1. **rGO only** (baseline spectrum).
2. **rGO + FAM-labeled aptamer** (to check binding interaction and quenching).
3. **rGO + FAM-aptamer + fentanyl** at **10 nM** and **20 nM** (to study displacement/release effect).
4. **FAM-labeled aptamer only** (to observe native aptamer absorbance).
5. **Fentanyl (50 nM) + FAM-labeled aptamer** (without rGO, to check direct binding effect).

Procedure

- Each sample was prepared in 100 µL volume using appropriate buffer (TE or Milli-Q).
- Samples were incubated at room temperature for 10 min in the dark.
- Absorbance spectra were recorded using a UV–Visible spectrophotometer in the range 200–800 nm.
- Baseline correction was performed using buffer alone.
- Particular attention was given to characteristic peaks:

- **rGO**: broad absorption in visible region.
- **FAM dye**: peak ~450–495 nm.
- **rGO–aptamer interaction**: possible quenching (reduced FAM peak).
- **Fentanyl addition**: expected restoration of FAM absorbance due to aptamer release.

Microplate UV–Vis / Fluorescence protocol

Overview: samples were prepared and measured in a 96-well plate at 25 °C. Measurements were made as absorbance scans (200–800 nm) and FAM fluorescence (Ex ≈ 495 nm, Em ≈ 530 nm) where indicated. All steps involving fentanyl were performed in a certified chemical hood and with institutional controlled-substance procedures followed.

Plate layout (example)

- Wells were arranged in triplicate for each condition.
- Blank wells (buffer only) and control wells (aptamer only, rGO only, fentanyl only) were included on every plate.
  Example columns:
- **Blank:** 200 µL assay buffer.
- **rGO alone:** e.g., 180 µL buffer + 20 µL rGO (adjust depending on working concentration).
- **rGO + Aptamer:** 178–198 µL buffer + 20 µL rGO + 2–22 µL aptamer (depending on target nM; adjust total to 200 µL).
- **rGO + Aptamer + Fentanyl:** 176–196 µL buffer + 20 µL rGO + 2–22 µL aptamer + 2–20 µL fentanyl (depending on needed concentration).
- **Aptamer alone:** 198–200 µL buffer + 2–20 µL aptamer.
- **Aptamer + Fentanyl:** 196–198 µL buffer + 2–20 µL aptamer + 2–20 µL fentanyl.

Sample preparation

1. **Buffer equilibration**
    - Plate wells were equilibrated to 25 °C in the plate reader or on the bench for ≥10 min prior to reading.
2. **rGO alone**
    - The rGO suspension was diluted into assay buffer to the working concentration and 200 µL was dispensed into each designated well.
3. **rGO + FAM-aptamer (no fentanyl)**
    - The folded FAM-aptamer working solution was added to rGO wells to the final indicated concentration (e.g., 10 nM or 20 nM) so that a final volume of 200 µL per well was achieved. The mixtures were gently mixed by pipetting and were incubated for 5–10 min at 25 °C to allow adsorption / equilibration.
4. **rGO + FAM-aptamer + fentanyl**
    - Fentanyl working solution was added to the rGO + aptamer pre-incubated wells to give the final fentanyl concentration (e.g., 10 nM or 20 nM). The final well volume was adjusted to 200 µL with assay buffer. The plate was gently mixed and incubated for a defined time (e.g., 5–20 min) prior to reading. (If methanol content exceeded ~1% v/v it was minimized by preparing aqueous intermediates; handling was done in hood.)
5. **Aptamer alone**
    - Folded aptamer working solutions were dispensed into wells (200 µL) and incubated at 25 °C for 5 min, then read.
6. **Aptamer + fentanyl (no rGO)**
    - Fentanyl was added to aptamer wells to the desired final concentrations; samples were equilibrated for 5–20 min at 25 °C before measurement.

Notes: volumes were chosen as 200 µL per well for good optical pathlength and reproducibility; smaller volumes (e.g., 100 µL) were used where plate reader sensitivity was higher.

Measurement

1. **Absorbance scan (UV–Vis)**
    - Buffer blanks were measured and subtracted from sample spectra.
    - Absorbance scans from 200–600 nm were recorded for each well.
    - Where available, pathlength correction was applied so that microplate OD values were normalized to a 1-cm pathlength; otherwise absorbance values were reported as measured with the plate geometry noted.
2. **Fluorescence (FAM)**
    - Fluorescence measurements were recorded using excitation near 495 nm and emission near 520 nm (set to instrument filter or monochromator settings).
    - Fluorescence values were blank-subtracted (buffer and rGO blanks as appropriate).
    - Fluorescence quenching by rGO and fluorescence recovery upon fentanyl addition were monitored.

Data handling & typical observables

- **Aptamer absorption peaks** at ~260 nm (nucleic acid) and **FAM absorbance** at ~495 nm were recorded and compared between conditions. The appearance, disappearance, or wavelength shift of these peaks.
- **Quenching** of FAM fluorescence by rGO was observed as decreased signal at 520 nm; **target binding** (fentanyl) was inferred from partial or full recovery of fluorescence relative to aptamer alone.
- **Replicates** (≥3) were averaged and standard deviation/standard error was reported. Statistical test(s) were applied where appropriate.

13] BSA + GO Aggregation, FRET Assay

Rationale

- **Graphene oxide (GO)** tends to aggregate in biological buffers.
- **Bovine Serum Albumin (BSA)** acts as a blocking/stabilizing agent to prevent nonspecific GO aggregation.
- In this assay, **GO + aptamer** complex is formed. When **fentanyl binds the aptamer**, the aptamer detaches, causing changes in GO aggregation that can be visually/optically detected.

Preparation

1. **BSA Main Stock (6.625 mg/mL)**
    - 265 mg BSA dissolved in 40 mL of 1× PBS (pH 7.4).
2. **Working Stock (WS, 20 µg/mL)**
    - 30 µL of main stock + 970 µL PBS.
3. **Dilutions from WS (Final Vol = 500 µL each):**

Table 13a: Concentrations of BSA

Final Conc. (µg/mL)	Volume of WS (µL)	Volume of PBS (µL)
	BSA	
1 µg/mL	62.5	437.5
5 µg/mL	125	375
10 µg/mL	250	250

Final Conc. (µg/mL)	Volume of WS (µL) BSA	Volume of PBS (µL)
15 µg/mL	375	125

Microplate Setup (100 µL final per well):

- 50 µL GO + 50 µL BSA dilution.
- Positive control: FITC–aptamer + GO.
- Blank: Buffer only.

Assay Steps

1. GO powder was dissolved in water, sonicated (10–15 min), and diluted to working concentrations (e.g., 1 mg/mL stock).
2. Different BSA concentrations (1–15 µg/mL) were prepared from the WS using PBS.
3. Equal volumes (50 µL GO + 50 µL BSA dilution) were mixed in black 96-well plate.
4. FITC–aptamer was added to designated wells to monitor fluorescence.
5. Fentanyl was introduced (5–20 nM) into aptamer–GO–BSA wells.
6. Plate was incubated at RT, protected from light.
7. Fluorescence and aggregation were read on **Victor Nivo** (Ex 495 / Em 530).

The principle:

· **GO + FAM-aptamer** → fluorescence quenched.

- **Fentanyl binds aptamer** → aptamer detaches → fluorescence recovery.
- **BSA** helps stabilize GO in buffer, reducing false aggregation.

14] Order-of-Addition Sequences rGO / BSA / Aptamer / Fentanyl

Common conditions (used):

- rGO: **0.10 mg/mL** (sonicated immediately before use)
- FAM-aptamer: **10 nM** and **20 nM** (separate blocks)
- BSA: **2%** (w/v or as used)
- Fentanyl: **10 nM** (final)
- Well final volume: **100 µL** (prepare 50 µL + 50 µL mixes so pipetting is consistent)
- Plate reader: **Victor Neo**, Ex = **495 nm**, Em = **530 nm** (top read, endpoint, medium gain)
- Incubation: room temp, **10–15 min**, protected from light
- Replicates: **triplicate** per condition

Sequence 1 — rGO → BSA → Aptamer → Fentanyl

Performed:

1. **50 µL rGO (0.10 mg/mL)** was added to each well.

2. **50 µL BSA (2%)** was added and wells were incubated **2–5 min** to block GO surface.
3. **50 µL of FAM-aptamer solution** (prepared so that adding 50 µL produces the final aptamer conc, e.g., aptamer-only 50 µL at 20 nM) was added and incubated **10 min** (adsorption expected minimal because surface blocked).
4. **Fentanyl (10 nM)** was added where required (either pre-mixed into the 50 µL aptamer solution or added as a small-volume spike with care) and incubated **10 min**.

Sequence 2 — Aptamer → Fentanyl → rGO → BSA

Performed:

1. **50 µL aptamer + fentanyl mix** (aptamer pre-incubated with 10 nM fentanyl for 5–10 min) was prepared and **50 µL** of this mix was added to each well.
2. **50 µL rGO (0.10 mg/mL)** was added to the aptamer:fentanyl wells and incubated **10 min**.
3. **50 µL BSA (2%)** was then added (if used) to the wells and incubated **2–5 min**.

Sequence 3 — Aptamer → rGO → BSA → Fentanyl

Performed:

1. **50 µL aptamer** was added to each well.
2. **50 µL rGO (0.10 mg/mL)** was added and mixture was incubated **10 min** to allow aptamer adsorption and quenching.
3. **50 µL BSA (2%)** was added and incubated **2–5 min** (test whether BSA can displace or block further adsorption).
4. **Fentanyl (10 nM)** was added last and incubated **10 min**, then read.

Rationale / expected: Aptamer is adsorbed and quenched by rGO; BSA may partially displace weakly-bound aptamer or stabilize aggregates. Addition of fentanyl last may cause **partial recovery** of fluorescence if aptamer desorbs.

Table 11a: Order-of-Addition Sequences rGO / BSA / Aptamer / Fentanyl

Seq 1 (A)	Seq 2 (B)	Seq 3 (C)	Controls
Seq1 (10 nM aptamer)	Seq2 (10 nM)	Seq3 (10 nM)	Blank
Seq1 (20 nM aptamer)	Seq2 (20 nM)	Seq3 (20 nM)	rGO only
Seq1 + no fentanyl	Seq2 + no fentanyl	Seq3 + no fentanyl	Aptamer only
Seq1 + fentanyl (10 nM)	Seq2 + fentanyl	Seq3 + fentanyl	Positive control

Example well recipes (keep total = 100 µL by preparing 50 µL mixes)

- **Aptamer-only 50 µL**: 50 µL aptamer at target concentration (10 or 20 nM).
- **rGO 50 µL**: 50 µL of 0.10 mg/mL rGO.
- **BSA 50 µL**: 50 µL of 2% BSA (or buffer containing BSA).
- **Aptamer + fentanyl mix**: prepare 50 µL containing aptamer at final desired concentration and fentanyl at 10 nM so that adding 50 µL yields final well concentrations without further additions.

Data recording & calculations

- RFU (aptamer only), RFU (rGO+aptamer), RFU (after fentanyl) were recorded in triplicate.
- % Quenching and % Recovery was calculated as:

$$\%\ \text{Quenching} = \frac{F_{\text{apt only}} - F_{\text{rGO+apt}}}{F_{\text{apt only}}} \times 100$$

$$\%\ \text{Recovery} = \frac{F_{\text{post-fentanyl}} - F_{\text{rGO+apt}}}{F_{\text{apt only}} - F_{\text{rGO+apt}}} \times 100$$

Safety precautions

- **FAM samples were protected from light.**
- **rGO was sonicated immediately before use to ensure dispersion.**
- **Small-volume fentanyl additions were made using accurate pipettes or pre-mixed into the 50 µL aptamer solution to avoid sub-µL pipetting.**
- **All work with fentanyl was performed in a fume hood with PPE; waste placed in opioid waste container.**

15] rGO Quenching Study with FAM labelled aptamer

Set 1: Preparation of 5

cmg/mL rGO stock (10.00 mL)

- **50.00 mg** rGO powder was weighed and dissolved in **10.00 mL** Milli-Q water.
- The suspension was **vortexed briefly** and **sonicated (≈40 kHz, 10–15 min, RT)** to obtain a homogenous stock.
- Large aggregates, if present, were removed by brief centrifugation and the supernatant was used.

Dilutions prepared (each final volume = 3.00 mL) — performed

Volumes were calculated using $C_1V_1 = C_2V_2$ with C_1 = 5 mg/mL (stock).

Table 15a: Preparation of different concentrations of rGO

Target conc. (mg/mL)	Stock (mL)	Water (mL)	Final vol (mL)
1.00	0.600	2.400	3.000
2.00	1.200	1.800	3.000
3.00	1.800	1.200	3.000
4.00	2.400	0.600	3.000

Set 2: Lower concentrations (direct dilutions prepared fresh)

- **These were included to observe quenching at very low rGO levels.**

Table 15b: Dilutions of rGO

Target conc. (mg/mL)	Source stock used
1.00	As above (from 5 mg/mL)
0.50	Diluted from 1 mg/mL stock (1:2 with Milli-Q)
0.20	Prepared from 1 mg/mL stock (1:5)
0.10	Prepared from 1 mg/mL stock (1:10)
0.05	Prepared from 1 mg/mL stock (1:20)

Performed: For each concentration, the required volume of 10 mg/mL stock was pipetted into a low-bind tube and Milli-Q water was added to 3.00 mL total. Each tube was gently inverted / vortexed and, if needed, briefly sonicated to re-disperse rGO.

Assay Setup

- Each rGO concentration (5, 4, 3, 2, 1, 0.5, 0.2, 0.1, 0.05 mg/mL) was incubated with a constant concentration of FAM-labelled aptamer.
- **Well volume:** 100 µL (50 µL rGO + 50 µL aptamer).
- Fluorescence quenching was measured on the Victor Neo plate reader. Readings were taken with Ex = 495 nm, Em = 530 nm, top-read endpoint mode, medium gain. Raw RFU values were recorded and blank values were subtracted before analysis.

- **Percent quenching** was calculated as:

$$\% \text{ Quenching} = \frac{F_{\text{aptamer only}} - F_{\text{rGO+aptamer}}}{F_{\text{aptamer only}}} \times 100$$

Storage & precautions

- Working stocks were kept at **4 °C** (short term) and protected from dust/contamination. Prior to use, stocks were gently vortexed or briefly sonicated.
- Low-bind tubes were used to minimize adsorption losses.
- Pipetting of volumes ≥0.6 mL (600 µL) was performed with calibrated pipettes; small transfers (≤10 µL) were avoided in subsequent plate preparations by making appropriate intermediates.

16] Fluorescence Optimization for FAM–Aptamer

Step 1: Excitation Scan

- Fixed **Emission = 530 nm**
- Varied excitation wavelength to find the best Ex.

Step 2: Emission Scan

- Fixed **Excitation = 495 nm** (from Step 1 result)
- Varied emission wavelength to find the best Em.

- Different excitation/emission filter sets (Ex: 355–540 nm; Em: 530–685 nm) were tested using **Victor Neo**.
- Aptamer concentrations: **10 nM and 20 nM** were screened.
- Maximum fluorescence signal with minimal background was obtained at:
  Excitation: 495 ± 20 nm
  Emission: 530 ± 30 nm
- This setting was used for all subsequent quenching studies with rGO and fentanyl.

Step 1: Excitation Scan (Emission fixed at 530 nm)

Excitation (Ex)	Emission (Em, fixed)	Bandwidth
355 / 40 nm	530 /30 nm	±30 nm
435 /20 nm	530 /30 nm	±30 nm

Excitation (Ex)	Emission (Em, fixed)	Bandwidth
460 /30 nm	530 /30 nm	±30 nm
480 /30 nm	530 /30 nm	±30 nm
495 /20 nm	530 /30 nm	±30 nm
540 /20 nm	530 /30 nm	±30 nm

Step 2: Emission Scan (Excitation fixed at 495 nm)

Excitation (Ex, fixed)	Emission (Em)	Bandwidth
495 /20 nm	540 /10 nm	±10 nm
495 /20 nm	580 /20 nm	±20 nm
495 /20 nm	625 /30 nm	±30 nm
495 /20 nm	**530 /30 nm**	±30 nm
495 /20 nm	640 /30 nm	±30 nm
495 /20 nm	685 /30 nm	±30 nm

17] FRET assay — rGO / FAM-aptamer / fentanyl

FRET (quenching & recovery) was assayed using FAM-labelled aptamer (10 nM) with fentanyl concentrations (5, 10, 15, 20 nM) and rGO test concentrations (1.0, 1.3, 1.5, 1.9 mg/mL). Test wells were assembled, initial quenching was monitored after a 15 min incubation (rGO + aptamer), then fentanyl was added and fluorescence recovery was monitored from 0 → 15 min.

Instrument / settings: Victor Neo plate reader — **Ex = 495 nm**, **Em = 530 nm**, top read, endpoint/time-course mode, medium gain. Samples protected from light.

Well compositions

Main experimental wells (pre-fentanyl):

- **60 µL** Milli-Q / buffer
- **20 µL** rGO (at chosen test conc: 1.0 / 1.3 / 1.5 / 1.9 mg/mL)
- **20 µL** FAM-aptamer (10 nM)
  → **Total = 100 µL**.
  These wells were incubated 15 min to allow adsorption / quenching.

Fentanyl addition (performed after initial incubation):

- After the 15 min incubation and initial read, **20 µL** of fentanyl working solution (prepared to give final 5 / 10 / 15 / 20 nM in the well after addition) was added to each test well.
  → **Final well volume after fentanyl addition = 120 µL.**

Controls (as performed):

- **Positive control (max fluorescence):** 80 µL water + **20 µL FAM-aptamer** (no rGO).
- **Blank:** water only.
- **rGO only:** 80 µL water + **20 µL rGO** (no aptamer) — checks rGO background.
- **Quenching check:** 60 µL water + 20 µL rGO + 20 µL FAM-aptamer (100 µL) — measured before fentanyl addition to record quenching baseline.

Practical note (as performed): to avoid sub-µL fentanyl pipetting, fentanyl working stocks were prepared at concentrations that allowed 20 µL addition to reach the desired final concentrations.

Timeline / measurement schedule

1. Plate wells were prepared with water, rGO and FAM-aptamer (see “Main experimental wells”).
2. Plate was gently mixed and **incubated 15 min at room temperature** (protected from light).
3. **Initial fluorescence read** was taken (this records the quenched signal after aptamer adsorption).
4. **20 µL fentanyl** (one of 5, 10, 15 or 20 nM final) was added to each test well. Time was set to **t = 0** at the moment of fentanyl addition.
5. Fluorescence was recorded at multiple time points to follow recovery: **0, 1, 2, 5, 10, 15 minutes** (or as otherwise needed).
6. Triplicates were measured for each condition.

Data recording & calculations

- **Raw RFU** were blank-subtracted (blank = water).
- **% Quenching** (before fentanyl) was calculated as:

$$\% \text{ Quenching} = \frac{F_{\text{aptamer only}} - F_{\text{rGO+apt}}}{F_{\text{aptamer only}}} \times 100$$

Where, Aptamer only = positive control, FrGO+apt = RFU after 15 min incubation (pre-fentanyl).

% Recovery (after fentanyl at time t) was calculated as:

$$\% \text{ Recovery}(t) = \frac{F_{\text{t}} - F_{\text{rGO+apt}}}{F_{\text{aptamer only}} - F_{\text{rGO+apt}}} \times 100$$

- where FtF_tFt = RFU at time t after fentanyl addition.

Record raw RFU, blank, % quenching and % recovery for each replicate and condition.

Safety & practical notes

- Fentanyl handling was performed in a certified fume hood with double gloves, respirator, goggles and dedicated opioid waste disposal. Small-volume additions were avoided by preparing intermediate stocks so that 20 µL additions delivered the desired final concentrations.
- FAM samples were protected from light. rGO stocks were briefly sonicated before use to re-disperse.
- If final well volumes varied between test and control wells (100 vs 120 µL), RFU comparisons were normalized to account for volume effects (or controls were prepared with matching volumes).

18] FRET Assay — GO / FAM-Aptamer / Fentanyl

- **Components**:
  - GO suspensions at working concentrations (50, 25, 12.5, 6.25, 3.125 µg/mL)
  - FAM-labelled aptamer: 10 nM
  - Fentanyl: 5, 10, 15, 20 nM
- **Controls**:
  - Blank (buffer only)
  - Positive control (Aptamer only)
  - GO only (without aptamer)
  - GO + Aptamer (quenching check)

- **Protocol**:
  - Each well (100 µL final volume):
    - 60 µL water/buffer
    - 20 µL GO suspension (variable concentration)
    - 20 µL FAM–aptamer (10 nM)
  - Plate incubated 15 min at room temperature, protected from light.
  - Fentanyl (5–20 nM) added after incubation, readings recorded from 0–15 min.
- **Instrument**:
  - Victor Nivo microplate reader
  - Excitation: 495 nm & Emission: 530 nm

19] FRET Assay — AuNP / FAM-Aptamer / Fentanyl

Experimental procedure

1. The 96-well black plate was pre-labelled; triplicates were used for each condition.
2. AuNP working stocks were vortexed briefly and equilibrated to room temperature. The appropriate volume of AuNP (per table above) was added to each well.
3. Milli-Q / Buffer A was added to each well according to the table so that 20 µL FAM addition gave 100 µL total.
4. **20 µL** of FAM-aptamer working solution was added to each well. The plate was mixed gently by pipetting and then incubated at room temperature for **10–15 min** to allow AuNP–aptamer interaction & quenching.
5. An initial fluorescence read (pre-fentanyl) was optionally taken to record the quenched signal (FITC channel).

6. **5 µL** of fentanyl stock (20× final) was added to each well to produce final fentanyl concentrations of **5, 10, 20, 25, 50, 75, 80 nM** (as required). Time = 0 was marked at the moment of fentanyl addition.
7. Fluorescence was recorded on the Victor Neo at timepoints **0, 1, 2, 5, 10, 15 min** (or a subset as required). Ex = 495 nm; Em = 530 nm; top read, medium gain. All readings were blank-subtracted.
8. Triplicate averages and standard deviations were calculated for each condition.

Controls (run on each plate)

- **Blank:** Buffer only.
- **FAM only (no AuNP):** maximum fluorescence reference.
- **AuNP only (no FAM):** checks scattering/background.
- **FAM + AuNP (no fentanyl):** quenching baseline.
- **Fentanyl only:** checks any direct fluorescence effect (rare).
- **Positive control:** FAM + known non-adsorbing particle or buffer if used.

Table: 18a Setup for AuNP, FAM Aptamer, Fentanyl FRET Assay

Sample	AuNP Volume (µL)	MilliQ (µL)	FAM (µL)	Final AuNP Conc.	Fentanyl Conc. Tested (nM)
Control	0	80	20	0 nM	0
S1	2	78	20	~1.5 nM	5, 10, 20, 25, 50, 75, 80
S2	5	75	20	~10 nM	5, 10, 20, 25, 50, 75, 80
S3	10	70	20	~20 nM	5, 10, 20, 25, 50, 75, 80
S4	20	60	20	~50 nM	5, 10, 20, 25, 50, 75, 80
S5	30	50	20	>50 nM (higher test)	5, 10, 20, 25, 50, 75, 80

Sample	AuNP Volume (µL)	MilliQ (µL)	FAM (µL)	Final AuNP Conc.	Fentanyl Conc. Tested (nM)
S6	50	30	20	>>50 nM (saturation)	5, 10, 20, 25, 50, 75, 80

Expected observations / interpretation

- AuNP addition produced **quenching** of FAM fluorescence (lower RFU vs FAM only).
- Fentanyl binding to the FAM-aptamer was expected to reduce aptamer adsorption to AuNP, leading to **partial or full fluorescence recovery**. The magnitude and kinetics of recovery were dependent on AuNP concentration and fentanyl concentration.
- Higher fentanyl concentrations were expected to yield faster and larger recovery.

20] Paper based Assay

Materials & reagents

- Whatman filter paper circles (pre-cut)
- Glass microscope slides (clean, labeled)
- 70% ethanol (or recommended sterilant)
- rGO suspension (1.5 mg/mL, sonicated immediately prior to use)

- FAM-labelled aptamer solution (10 nM)
- Fentanyl solution (20 nM, prepared in fume hood per SOP)
- TE buffer, 1× (room temperature)
- Pipettes and low-retention tips (P2, P20)
- Forceps, tweezers, tissue paper, gloves, eye protection, lab coat, respirator if required
- UV lamp for fluorescence observation (FAM channel: Ex ≈ 495 nm, Em ≈ 530 nm)

Safety

- All fentanyl handling was performed in a certified chemical fume hood.
- Double gloves, lab coat, eye protection, and respirator were worn.
- Opioid waste containers were used for tips/tubes; a naloxone kit was available.
- FAM solutions and slides were protected from light when not imaged.

Slide Preparation

1. Gloves and PPE were donned; the bench area was covered with absorbent paper.
2. Circular Whatman discs were affixed to labeled microscope slides using sterile forceps.
3. Slides were surface-sterilized with 70% ethanol and allowed to air dry.

Assay Additions & Imaging

All additions were 5 µL unless noted. Pre-mixing outside the slide was used to maintain consistent volumes.

1. **rGO application**
    - 5 µL rGO (1.5 mg/mL) was deposited centrally on each paper disc.
    - Slides were allowed to sit 1–2 min for adsorption.
    - Fluorescence was observed under UV lamp; photograph taken.
2. **FAM-aptamer addition**
    - 5 µL FAM-aptamer (10 nM) was added on rGO spot.
    - Incubation for 2–5 min was performed.
    - Fluorescence was observed under UV; photograph taken.
3. **Fentanyl addition**
    - 5 µL fentanyl (20 nM) was added to the same spot in fume hood.
    - Incubation of 0–15 min; fluorescence monitored at defined timepoints (0, 1, 5, 10, 15 min).
    - Photographs were taken at each timepoint.
4. **TE buffer addition (control)**
    - 5 µL TE buffer (1×) was added; fluorescence observed under UV after ~2 min.

Controls

- Paper disc + buffer only (blank)
- Paper disc + FAM-aptamer only (no rGO)
- Paper disc + rGO + aptamer (no fentanyl)
- Paper disc + rGO + fentanyl (no aptamer)

Notes

- Slides were discarded if rGO spread beyond paper disc.
- Sonicated rGO stock was re-prepared if uneven spots or air bubbles were observed.
- FAM images were captured quickly; slides were covered between imaging steps.
- Additional timepoints up to 30 min were collected if fluorescence recovery was slow.

Example Experimental Parameters

- rGO: 1.5 mg/mL, 5 µL/spot
- FAM-aptamer: 10 nM, 5 µL/spot
- Fentanyl: 20 nM, 5 µL/spot
- TE buffer: 1×, 5 µL/spot
- Incubation: 1–2 min after rGO; 2–5 min after aptamer; 0–15 min after fentanyl
- UV-lamp fluorescence: Ex ≈ 495 nm, Em ≈ 530 nm
-