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1]. Nanoparticle Synthesis and Characterization (Gold Nanoparticles) for nanoparticle-based sensing

Gold nanoparticles (AuNPs) were synthesized by the reduction of chloroauric acid ($HAuCl_4$) by trisodium citrate in aqueous solution. In this process, citrate functions as both a reducing agent and a stabilizing/capping ligand, preventing particle aggregation and controlling nanoparticle size.

Materials and Reagents

I. Chloroauric acid trihydrate ($HAuCl_4 \cdot 3H_2O$, analytical grade)
II. Trisodium citrate dihydrate ($Na_3C_6H_5O_7 \cdot 2H_2O$, analytical grade)
III. Ultrapure water (Milli-Q grade)
IV. Sodium borohydride ($NaBH_4$, 0.1 M, freshly prepared)

Equipment

I. Clean borosilicate glassware (250 mL beaker or round-bottom flask)
II. Magnetic stirrer with heating capability
III. Magnetic stir bar (Teflon-coated)
IV. Volumetric flasks and pipettes
V. Thermometer
VI. UV-Visible spectrophotometer for characterization

Steps

1. Prepare 1% (w/v) trisodium citrate stock solution by dissolving 1.00 g of $Na_3C_6H_5O_7 \cdot 2H_2O$ in 100.0 mL ultrapure water.
2. Calculate and prepare 0.25 mM $HAuCl_4$ solution (8.50 mg $HAuCl_4 \cdot 3H_2O$ in 100.0 mL water for 100 mL final volume).
3. Clean all glassware thoroughly with laboratory detergent, followed by extensive rinsing with tap water and ultrapure water.
4. Ensure complete removal of contaminants as colloidal systems are highly sensitive to impurities.

5. Add 50 mL of 0.25 mM $HAuCl_4$ solution and 0.25 mM trisodium citrate to a clean 250 mL vessel.
6. Place magnetic stir bar and begin continuous stirring.
7. Add 0.6 mL of freshly prepared, ice-cold 0.1 M $NaBH_4$ solution dropwise while maintaining vigorous stirring.
8. Observe immediate color change from pale yellow to deep red, indicating AuNP formation.
9. Continue stirring for 15 minutes at room temperature to ensure complete reduction and particle stabilization.
10. Monitor the solution for uniform deep red coloration characteristic of ~20 nm AuNPs.
11. Transfer the synthesized AuNPs to clean amber glass vials.
12. Store at 4°C for long-term stability (weeks to months).

Characterization

1. Transmission Electron Microscopy (TEM) revealed spherical gold nanoparticles with an average diameter of 10 ± 2 nm.

2. Scanning Electron Microscopy (SEM) and TEM analyses confirmed uniform dispersion of nanoparticles with sizes ranging from 8 to 12 nm.

3. UV–Visible spectroscopy displayed a characteristic surface plasmon resonance band at approximately 520 nm, corroborating the successful formation of gold nanoparticles.

Tips & troubleshooting

1. **Insufficient Reduction (Pale/Yellow Solution):**

Verify that the trisodium citrate solution is freshly prepared and that the reaction mixture was at a vigorous boil when citrate was introduced. Incomplete reduction may also result from aged sodium borohydride; prepare the $NaBH_4$ solution immediately prior to use and maintain it ice-cold during addition.

2. **Aggregation or Overgrowth (Blue/Gray Solution, Visible Aggregates):**

Contaminants accelerate aggregation. Ensure all glassware and ultrapure water are free of impurities. Reduce the citrate-to-gold molar ratio to limit particle growth. Aggregates can be removed by centrifugation (8,000–10,000 rpm for 10 minutes) followed by gentle resuspension in fresh citrate solution.

3. **Control of Particle Size:**

For smaller nanoparticles (~10 nm), increase the citrate-to-gold ratio (e.g., add 2–3 mL of 1% citrate stock to 100 mL of 0.25 mM $HAuCl_4$) to promote rapid nucleation and yield smaller diameters.

For larger nanoparticles (>20 nm), decrease the citrate-to-gold ratio or employ a seed-mediated growth approach to favor growth over nucleation.

4. **pH Considerations:**

Maintain the reaction pH between 4 and 7. Solutions with pH < 4 can inhibit citrate's reducing capacity, whereas pH > 7 may destabilize the colloid and broaden the size distribution. Monitor pH using pH paper or a calibrated meter.

5. **Storage and Monitoring:**

Store AuNP suspensions at 4 °C in amber glass vials to minimize photodegradation. Avoid freeze–thaw cycles. Periodically assess colloidal stability via UV–Vis spectroscopy; a sharp surface plasmon resonance peak at 520–525 nm indicates monodispersity, whereas peak broadening or red-shifting signals aggregation

.

Safety

- Handle chloroauric acid ($HAuCl_4$) in a chemical fume hood while wearing nitrile gloves, safety goggles, and a lab coat, as it is a strong oxidizer and toxic if ingested or inhaled.
- Although trisodium citrate poses low hazard, standard laboratory personal protective equipment (PPE) including gloves and eye protection should be employed.

- Exercise caution when handling hot liquids; use heat-resistant gloves and appropriate tools to prevent thermal injury.
- Collect all gold-containing waste in designated hazardous metal waste containers and dispose of it in accordance with institutional hazardous waste management protocols.

2]. Preparation and Characterization of Fluorescence Resonance Energy Transfer (FRET) Fluorophore and Quencher molecule Graphene Oxide (GO) Suspension

Procedure

I. Accurately weigh 1.00 mg of graphene oxide (GO) powder.
II. Transfer the GO into a clean glass vial and add 20.0 mL of Milli-Q water, yielding a 0.05 mg/mL suspension.
III. Sonicate the suspension at 40 kHz for 30 minutes at room temperature to achieve uniform dispersion of GO sheets.
IV. Measure the UV–Visible absorbance of the GO suspension over the 200–800 nm wavelength range using a calibrated spectrophotometer.
V. Record the characteristic absorption peak observed near 450 nm, confirming the presence of dispersed GO in solution.

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
12.5	1:4	250	750	1000
6.25	1:8	125	875	1000
3.125	1:16	62.5	937.5	1000

3]. 1. Preparation of Standard Dye Fluorescein Isothiocyanate (FITC) Stock Solutions and Serial Dilutions

FITC 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

DMSO stock preparation-

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

ii. Add **5 mL anhydrous DMSO** (molecular biology grade).

iii. Result: **1 mg/mL (2.57 mM) FITC in DMSO**.

iv. Mix: brief vortex (5–10 s) → gentle flick → spin down. If particulates remain, repeat; optionally **0.22 µm PTFE** filter (amber syringe).

v. **Aliquot** (e.g., 50–100 µL) into amber tubes, flush headspace with inert gas if available.

vi. **Storage:** −20 °C (or −80 °C for long term), protected from light. Avoid >3 freeze–thaw cycles.

vii. **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.

Aqueous working stock preparation: 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
80	2000	16	1984
60	2000	12	1988
40	2000	8	1992
20	2000	4	1996

4]. Fluorescence Resonance Energy Transfer (FRET) Mechanism and Theoretical Framework for Detection of Opioid – Fentanyl

Interaction & adsorption: FITC molecules adsorb onto graphene oxide (GO) surfaces via π–π stacking and hydrophobic/ electrostatic interactions. Adsorption brings the fluorophore into close 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).

Quenched state : 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). write in scitenfic and properly.

5]. Establishment of FRET Pair and Conjugation for the detection of Fentanyl.

1. Graphene Oxide-Fluorescein Isothiocyanate (GO-FITC) Fluorescence Quenching Assay

GO was selected as the acceptor molecule due to its quenching property in pair with FITC selected as the donor pair for fluorescence-based resonance energy transfer.
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 commencing the assay.
2. Into each test well, 50 µL of graphene oxide (GO) stock solution ranging from 3.125 to 50 µg/mL was dispensed. For the GO-only control wells, 50 µL of GO stock and 50 µL of buffer were added.
3. Subsequently, 50 µL of each fluorescein isothiocyanate (FITC) working stock solution (20–100 nM) was added to the designated wells. FITC-only control wells received 50 µL of FITC stock and 50 µL of distilled water. All solutions were handled in separated blocks to minimize the risk of cross-contamination.
4. The plate contents were gently mixed either by pipetting up and down once or by brief shaking, with care taken to avoid bubble formation. The plate was kept protected from light throughout mixing and subsequent steps.

5. Samples were incubated at ambient temperature for 10 to 15 minutes to facilitate adsorption and equilibration.
6. Fluorescence measurements were performed using a Victor Nivo plate reader with the following parameters: excitation wavelength of 495 nm, emission wavelength of 530 nm, top-read mode, endpoint mode with a total well volume of 100 µL, and medium gain settings. Raw relative fluorescence unit (RFU) values were recorded, and background fluorescence from blanks was subtracted.
7. Finally, the data were exported and graphed to compare fluorescence intensity between FITC-only wells and GO+FITC wells, enabling quantification of fluorescence quenching.

Percentage quenching as:

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

where

- $F_{\text{FITC only}}$ **is the fluorescence intensity of FITC alone (without quencher),**
- $F_{\text{GO+FITC}}$ **is the fluorescence intensity of the FITC in the presence of graphene oxide (quencher).**

- **Key:**
- Blank: Wells containing distilled water only.
- PC: Positive Control wells containing FITC only, without graphene oxide (GO).
- Exp: Experimental wells containing combinations of graphene oxide (GO) and FITC.
-

	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 containing distilled water are positioned at the edges of the plate for background signal subtraction.
- Positive controls with FITC only are included for each FITC concentration ranging from 20 to 100 nM.
- Experimental wells cover all possible combinations of five FITC concentrations and four graphene oxide (GO) concentrations, totaling 20 unique conditions.
- The remaining wells in rows D to H are reserved for replicate samples or additional experimental conditions as needed.

Notes & precautions

- Solutions and microplates containing fluorescein isothiocyanate (FITC) should be shielded from light exposure to prevent photobleaching and degradation.
- It is essential to run FITC-only control samples at final working concentrations (post-mixing) for accurate calculation of quenching percentages. An alternative approach involves measuring wells containing FITC alone, prepared with an equivalent volume plus buffer.
- In cases of weak or inconsistent fluorescence signals, briefly sonicate the graphene oxide (GO) stock solution (40 kHz, short duration) before use to ensure proper dispersion. Employ low-binding plates and tubes to minimize analyte adsorption losses.
- Document all plate reader settings, including gain and other instrument parameters, to enable reproducibility of fluorescence measurements.
- Dispose of waste containing graphene oxide and FITC following institutional hazardous waste management protocols.

6] Preparation of Working Stocks and Dilutions of Bovine Serum Albumin (BSA) in Phosphate-Buffered Saline (PBS)

Main Stock Preparation

I. A primary stock solution was prepared by accurately weighing 265 mg of bovine serum albumin (BSA).

II. The BSA was subsequently dissolved in 40 mL of 1× phosphate-buffered saline (PBS) at 100 mM concentration to formulate the main stock solution.

Working Stock preparation- 20 µg/mL (200 nM)

A working stock solution of 20 µg/mL (200 nM) was prepared by diluting 30 µL of the primary stock solution (super stock) with 970 µL of phosphate-buffered saline (PBS).

Preparation of Dilutions (500 µL each from Working Stock)

4. From the working stock, the following dilutions were made:

Table 6c: various PBS concentrations.

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

5. Each well was loaded with 50 µL of graphene oxide (GO) solution at the desired test concentration.
6. An additional 50 µL of the prepared BSA + PBS dilution was added to the same well, or solely PBS was added for the control wells.
7. The total volume in each well was 100 µL.

7]. 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
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

1. Preparation of Stock Solutions

i. Dissolve rGO powder in Milli-Q water to obtain a 1.0 mg/mL stock suspension. Sonicate for 10–15 minutes at room temperature to ensure uniform dispersion.
ii. Prepare FITC stock by dissolving 5 mg FITC in 5 mL water (≈2.56 mM).
iii. Generate a 1.0 mM FITC working stock by diluting 1.56 µL of 2.56 mM FITC stock into 3,998 µL water.
iv. Prepare FITC working solutions of 20, 40, 60, 80, and 100 nM by appropriate serial dilutions from the 1.0 mM working stock (e.g., mix 40 µL FITC stock with 1,960 µL water to yield 20 nM).

2. Fluorescence Quenching Assay Protocol for Reduced Graphene Oxide-FITC Interactions

i. Pipette 50 µL of rGO suspension at each test concentration into designated wells; for FITC-only controls, add 50 µL assay buffer instead.
ii. Add 50 µL of each FITC working solution to the respective wells.
iii. Gently mix by pipetting up and down once, taking care to avoid bubble formation.
iv. Protect the plate from ambient light throughout assembly and incubation.

3. Incubation

Incubate at room temperature for 15 minutes to allow FITC adsorption onto rGO and signal equilibration.

4. Fluorescence Measurement

i. Measure fluorescence using a Victor Nivo multimode plate reader under the following settings:
ii. Excitation: 495 nm
iii. Emission: 530 nm
iv. Read mode: Top-read, endpoint
v. Well volume: 100 µL
vi. Gain: Medium
vii. Record raw RFU values and subtract blank readings.

5. Data Analysis

Define FITC-only wells as 100% fluorescence reference.

Calculate percentage quenching for each rGO–FITC condition using:

$$\%\text{Quenching} = \frac{F\text{FITC only} - F\text{rGO+FITC}}{F\text{FITC only}} \times 100$$

Perform statistical analysis on blank-corrected data to determine quenching efficiency.

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 was prepared with a total volume of 100 µL (50 µL rGO suspension + 50 µL FITC solution).
- Columns 1–5 contained FITC-only controls.
- Column 6 served as the positive control with 20 nM FITC.
- Columns 7–13 comprised rGO quenching assays with a constant FITC concentration of 20 nM.
- Column 14 was designated as the blank (Milli-Q water or assay buffer).
- Rows A–C represented technical triplicates for each condition.

8] . Preparation and Standardization of Aptamer Stock Solutions

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-labelled)**: 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: $V1=C_2V_2/C_1$

$=10\ nM \times 2000\ \mu L/100000\ nM$

$= 0.2\ \mu L$ 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.

9]. Fentanyl Standard Solution Preparation and Concentration Optimization

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
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.

10]. UV-Visible Spectrophotometric Characterization and Analysis

i. Reduced graphene oxide (rGO) only (baseline spectral measurement).

ii. rGO with FAM-labeled aptamer (assessment of aptamer binding and fluorescence quenching).

iii. rGO with FAM-labeled aptamer and fentanyl at 10 nM and 20 nM (evaluation of fentanyl-induced aptamer desorption and fluorescence recovery).

iv. FAM-labeled aptamer only (native aptamer fluorescence profile).

v. Fentanyl (50 nM) with FAM-labeled aptamer, without rGO (assessment of direct aptamer–fentanyl interaction).

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.

Fluorescence Measurement Protocol

- **Overview**
  Samples were prepared in a 96-well plate and equilibrated at 25 °C. Absorbance spectra were recorded from 200 to 800 nm. FAM fluorescence measurements used excitation at ~495 nm and emission at ~530 nm. All procedures involving fentanyl were conducted within a certified chemical hood under institutional controlled-substance regulations.
- **Plate Layout**
  Wells were arranged in technical triplicate for each experimental condition. Blank and control wells were included on every plate:

– Blank: 200 µL assay buffer only.
– rGO alone: 180 µL buffer + 20 µL rGO suspension (adjust volume to achieve desired rGO concentration).
– rGO + Aptamer: 178–198 µL buffer + 20 µL rGO + 2–22 µL FAM-labeled aptamer (volumes adjusted to reach target aptamer concentration); total volume 200 µL.
– rGO + Aptamer + Fentanyl: 176–196 µL buffer + 20 µL rGO + 2–22 µL aptamer + 2–20 µL fentanyl (volumes adjusted to reach target concentrations); total volume 200 µL.
– Aptamer alone: 198–200 µL buffer + 2–20 µL aptamer.
– Aptamer + Fentanyl: 196–198 µL buffer + 2–20 µL aptamer + 2–20 µL fentanyl**.**

- **Measurement Steps**

1. Pipette samples into the designated wells in triplicate, seal the plate, and protect from light.
2. For UV–Vis scans, record absorbance across 200–800 nm.
3. For fluorescence, set excitation to 495 nm and emission to 530 nm; use top-read, endpoint mode.
4. Subtract blank values from all readings prior to data analysis.

Sample Preparation

i. **Buffer Equilibration**- Plate wells were equilibrated to 25 °C for at least 10 minutes, either within the plate reader or on the laboratory bench, prior to sample loading.

ii. **rGO** - The reduced graphene oxide suspension was diluted in assay buffer to the desired working concentration. A volume of 200 µL was dispensed into each designated well.

iii. **rGO + FAM-Aptamer (No Fentanyl)-** Folded FAM-labeled aptamer working solutions were added to the rGO-containing wells to reach the specified final aptamer concentration (e.g., 10 nM or 20 nM), adjusting volumes to a total of 200 µL per well. Each mixture was gently mixed by pipetting to avoid bubble formation and incubated at 25 °C for 5–10 minutes to allow aptamer adsorption and equilibration.

iv. **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.)

v. **Aptamer** - FAM-labeled aptamer working solutions (200 µL per well) were dispensed into designated wells and incubated at 25 °C for 5 minutes prior to fluorescence measurement.

vi. Aptamer + Fentanyl (No rGO)- Fentanyl stock solutions were added to the aptamer-containing wells to achieve the target final concentrations. Samples were equilibrated at 25 °C for 5–20 minutes before recording fluorescence readings.

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.

11]. Bovine Serum Albumin-Graphene Oxide Aggregation and FRET-Based Detection

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

12]. Order-of-Addition Optimization assay (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.**

13]. Fluorescence Quenching Assay FAM-Labeled Aptamers with Reduced Graphene Oxide

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

Target conc. (mg/mL)	Stock (mL)	Water (mL)	Final vol (mL)
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 prior to 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.

14]. Optimization of Excitation and Emission Parameters for FAM-Labeled Aptamer Fluorescence Assay

i. An excitation scan was performed with the emission wavelength fixed at 530 nm, varying excitation from 355 to 540 nm to determine the optimal Ex.
ii. An emission scan was conducted with the excitation wavelength set to 495 nm, varying emission from 530 to 685 nm to identify the optimal Em.
iii. Both 10 nM and 20 nM FAM–aptamer concentrations were evaluated using multiple filter sets (Ex: 355–540 nm; Em: 530–685 nm).
iv. The highest signal-to-background ratio was achieved at Ex 495 ± 20 nm and Em 530 ± 30 nm.
v. These optimized excitation and emission settings were applied to all subsequent rGO–FAM–aptamer quenching and fentanyl displacement assays.

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
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

15]. FRET Quenching and Recovery Assay Using FAM-Labeled Aptamer, rGO, and Fentanyl

i. FAM-labeled aptamer (10 nM) and reduced graphene oxide (rGO) at 1.0, 1.3, 1.5, and 1.9 mg/mL were used to assess fluorescence quenching and subsequent recovery upon fentanyl addition.

ii. Test wells were prepared with rGO and aptamer, incubated for 15 minutes at 25 °C, and initial fluorescence quenching was recorded.

iii. Fentanyl was then added to final concentrations of 5, 10, 15, and 20 nM; fluorescence recovery was monitored continuously over a 0–15-minute time course.

iv. Measurements were performed on a Victor Nivo plate reader in top-read, endpoint/time-course mode with excitation at 495 nm, emission at 530 nm, and medium gain.

v. All assays were protected from light to prevent photobleaching of the fluorophore.

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).

16] FRET-Based Assay Using Graphene Oxide, FAM-Labeled Aptamer, and Fentanyl

Components:

- GO suspensions prepared at working concentrations of 50, 25, 12.5, 6.25, and 3.125 µg/mL.
- FAM-labeled aptamer at a fixed concentration of 10 nM.
- Fentanyl at concentrations of 5, 10, 15, and 20 nM.

Controls:

- Blank control containing assay buffer only.
- Positive control with aptamer alone (no GO).
- GO only control (without aptamer) to assess background fluorescence and nonspecific effects.
- GO + aptamer wells for monitoring fluorescence quenching efficiency prior to fentanyl addition.

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

17]. FRET-Based Assay Using Gold Nanoparticles, FAM-Labeled Aptamer, and 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 in 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
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.

18]. Development of Paper-Based Analytical Device for Point-of-Care Detection

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

Experiment: Visualization of Fentanyl and Aptamer on Whatman Paper Using FRET Pair

Objective: To visualize the interaction between fentanyl and FAM-labeled aptamer on Whatman paper using rGO FRET pairs and optimize fluorescence signal.

Materials:

Whatman paper

Reduced graphene oxide (rGO)

FAM-labeled aptamer (10 nM and 500 nM)

Fentanyl (10 nM and 500 nM)

Gold nanoparticles (AuNPs)

IVIS Bioimaging system

Micropipettes, tips, and standard lab equipment

Methodology:

Preparation and Optimization of FRET-Based Assay on Whatman Paper

Whatman paper was cut into small, manageable pieces, and a depression was created on each piece to retain assay reagents.

Initial Assay:

a. mg of reduced graphene oxide (rGO) was deposited into the depression on the paper.
b. 1 µL of 10 nM FAM-labeled aptamer and 1 µL of 10 nM fentanyl were added sequentially.
c. The samples were air-dried, and imaging was performed using an IVIS Bioimaging system. No appreciable fluorescence signal was detected.

Volume Variation Optimization:

Reaction volumes were varied while maintaining constant reagent concentrations, but fluorescence remained very weak.

Concentration Optimization:

a. Aptamer concentration was increased to 500 nM, and various reaction volumes (1 µL to 6 µL) were tested.
b. Imaging confirmed fluorescence, indicating improved signal with higher aptamer concentration.

Optimized Assay Conditions:

a. The optimal assay involved 1 µL of 500 nM FAM-labeled aptamer and 1 µL of 500 nM fentanyl in the presence of rGO.
b. Fluorescence was successfully visualized using bioimaging techniques.

Comparison with Gold Nanoparticles (AuNPs):

a. Subsequent assays replaced rGO with AuNPs, which yielded significantly enhanced fluorescence signals.
b. Comparative experiments demonstrated superior fluorescence with the AuNP system over rGO.

Fluorescence Microscopy and Image Analysis:

a. Fluorescence microscopy was used to capture images of the assays.
b. ImageJ software was utilized for quantitative analysis of fluorescence intensity.

Observations:

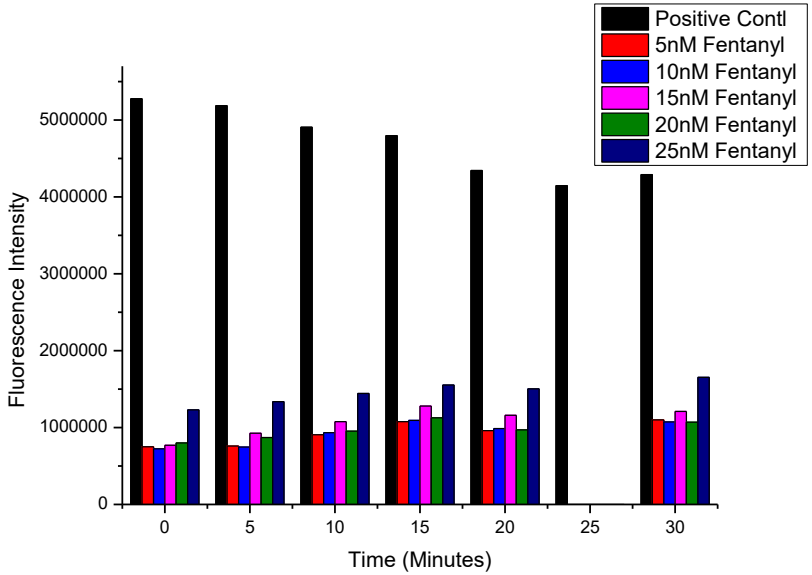
a. Low aptamer concentration (10 nM) resulted in negligible fluorescence, while increasing concentration and careful volume optimization markedly improved the signal.
b. AuNP-based assays consistently produced stronger fluorescence compared to rGO-based assays.
c. Analysis confirmed the effective FRET-based visualization of fentanyl-aptamer interactions on paper substrate.

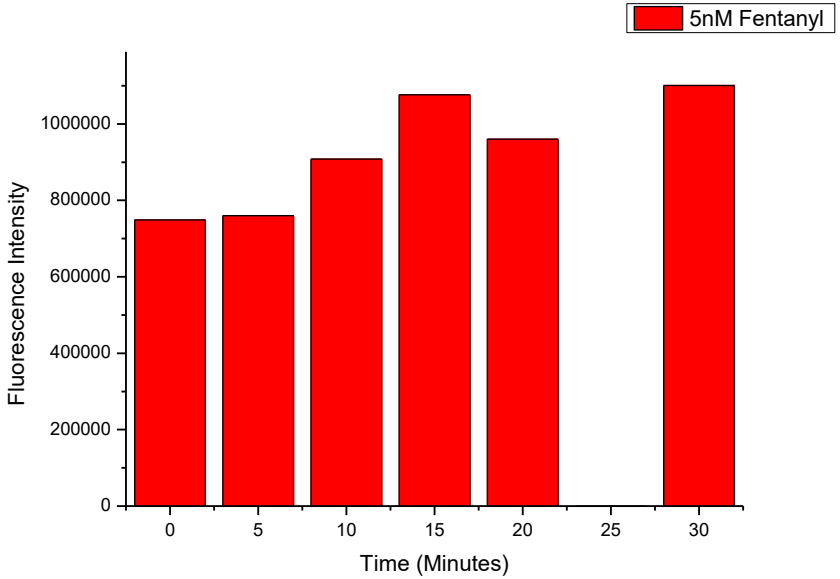
Conclusion:

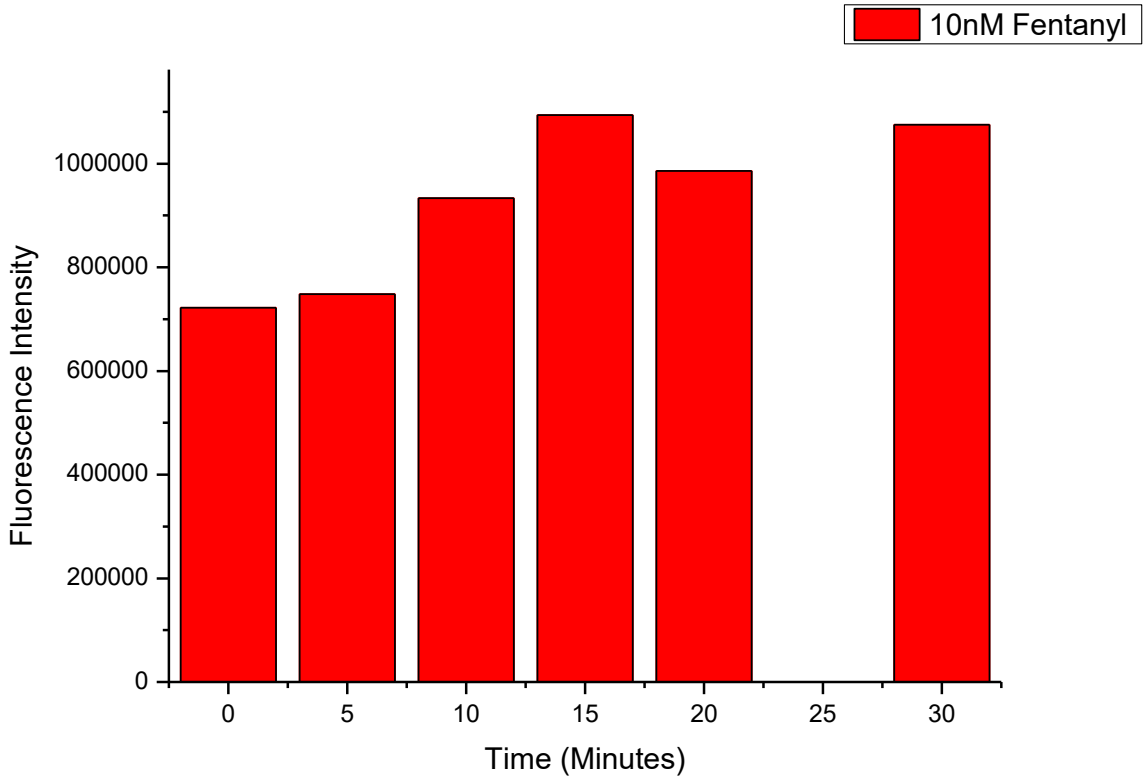
a. **FRET-based fentanyl detection on Whatman paper is feasible but requires optimization of aptamer concentration and reagent volumes.**
b. **The use of gold nanoparticles markedly enhances fluorescence signal quality.**
c. **The optimized protocol employing 500 nM FAM-labeled aptamer and fentanyl (1 µL each) with AuNPs is established as a reliable platform for future detection experiments.**

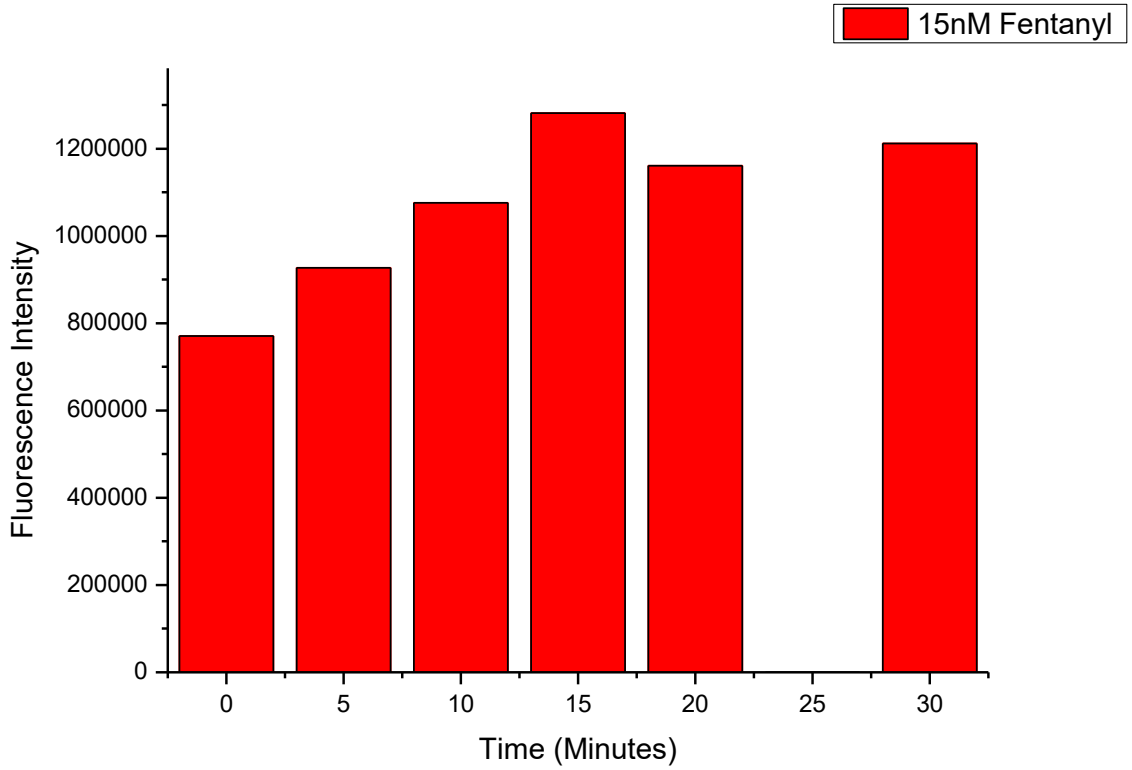
Results and Graphical analysis:

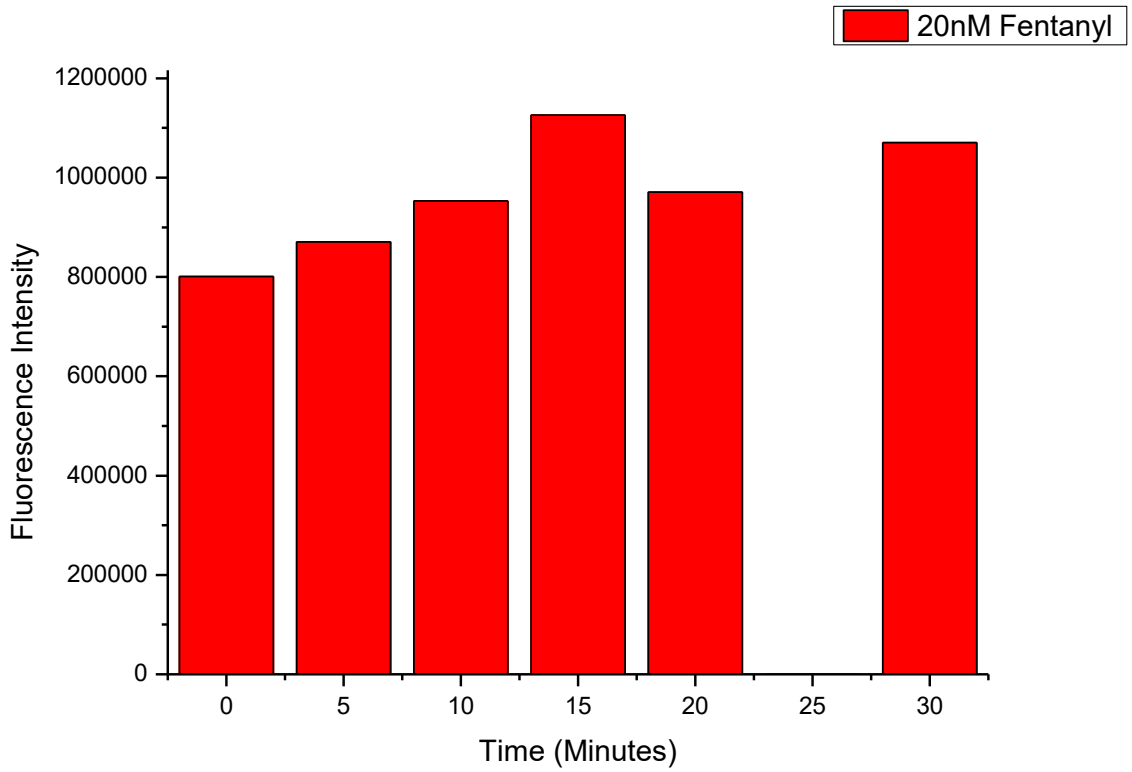
FRET Assay with Fentanyl (5nM to 25nM)

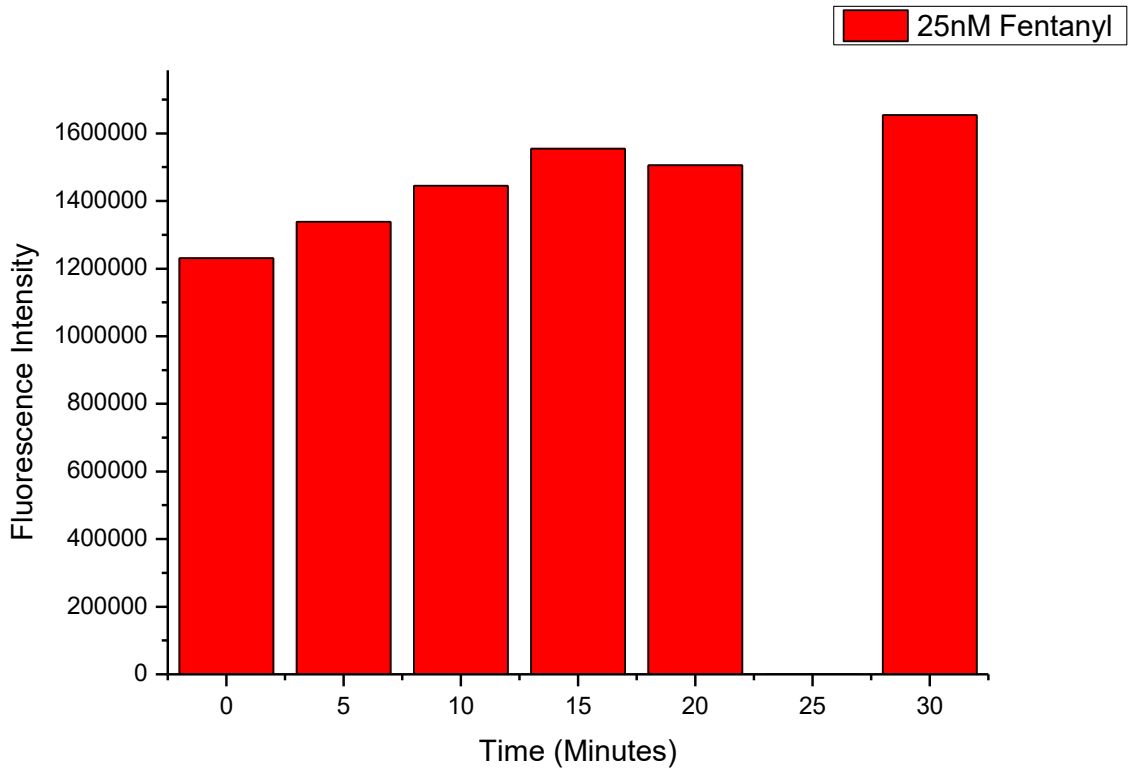


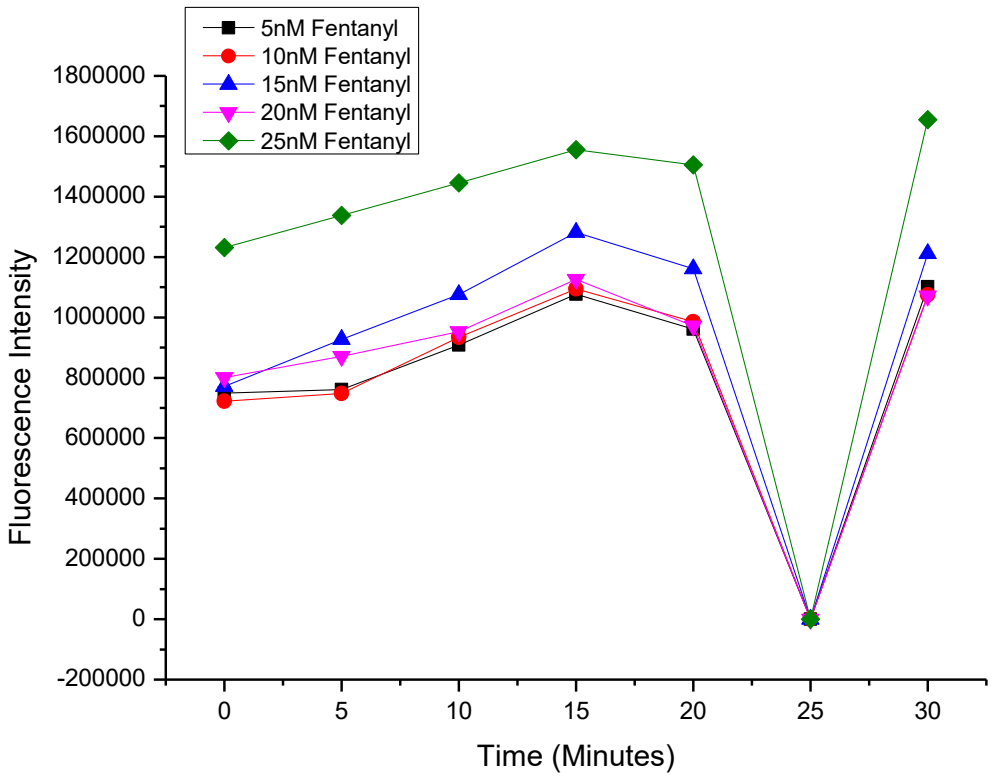



10nM Fentanyl
Fluorescence Intensity
1000000
800000
600000
400000
200000
0
0
5
10
15
20
25
30
Time (Minutes)


15nM Fentanyl
Fluorescence Intensity
0
200000
400000
600000
800000
1000000
1200000
0
5
10
15
20
25
30
Time (Minutes)

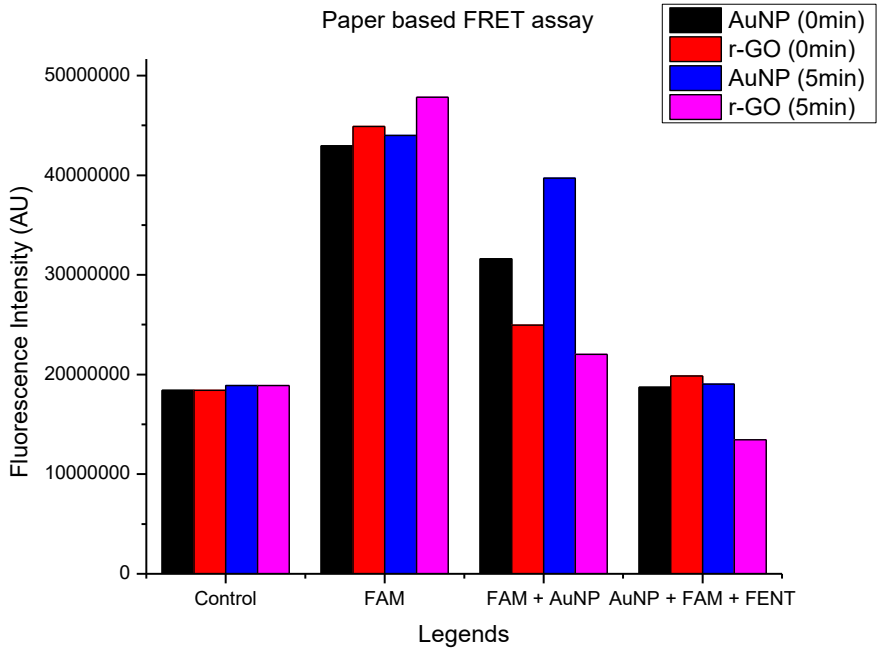

20nM Fentanyl
Fluorescence Intensity
1200000
1000000
800000
600000
400000
200000
0
0
5
10
15
20
25
30
Time (Minutes)

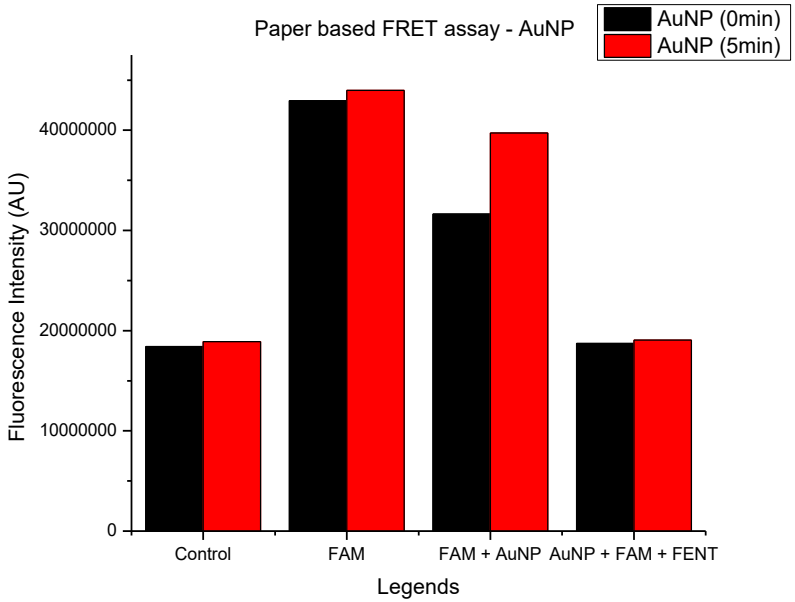

25nM Fentanyl
Fluorescence Intensity
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
0
5
10
15
20
25
30
Time (Minutes)

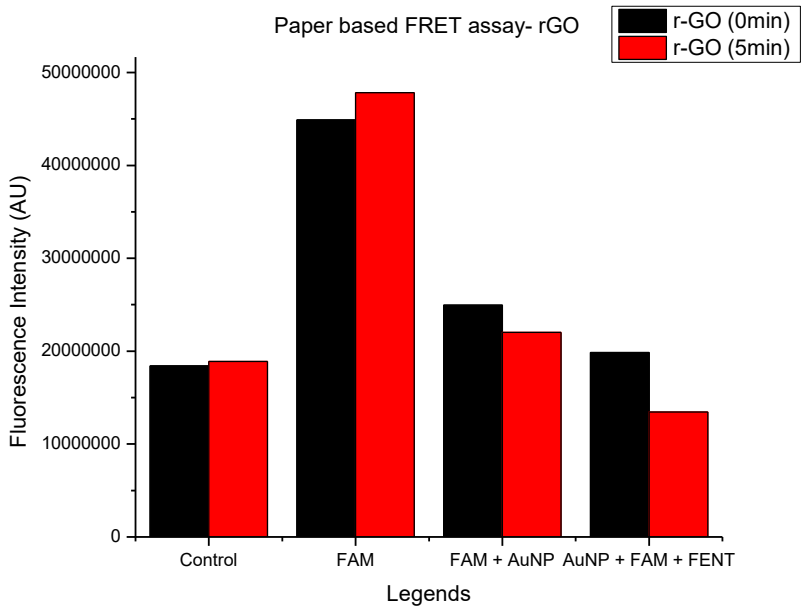

5nM Fentanyl
10nM Fentanyl
15nM Fentanyl
20nM Fentanyl
25nM Fentanyl
Fluorescence Intensity
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
0
-200000
0
5
10
15
20
25
30
Time (Minutes)

Paper-based assay results:

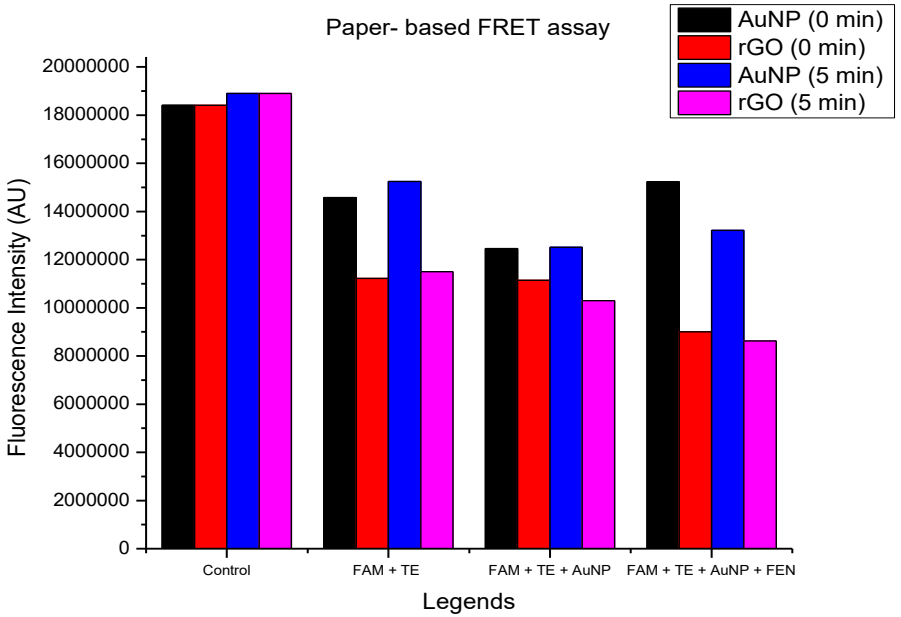
AuNP and RGO assays with Fentanyl: SET 1:

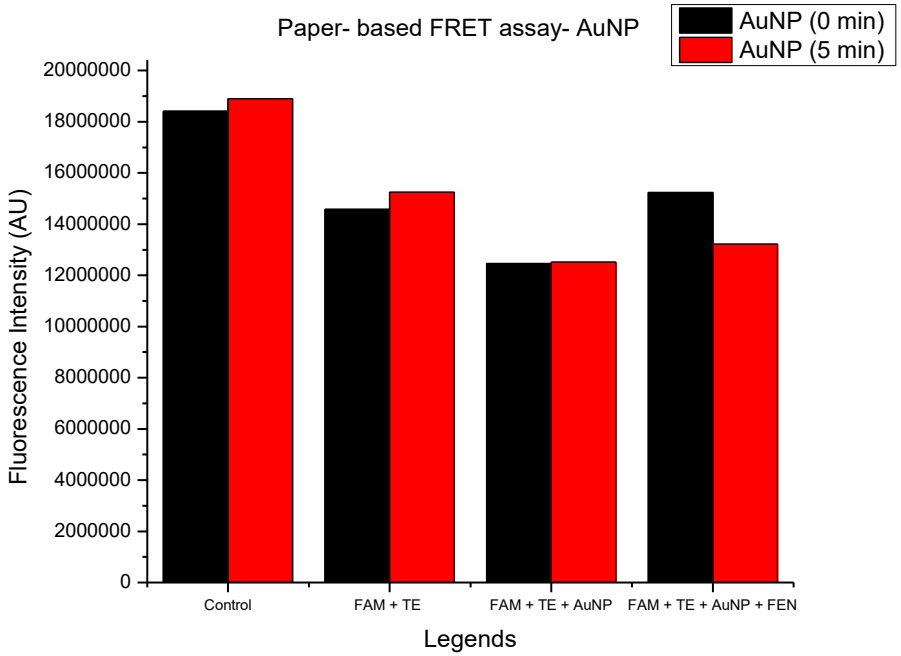


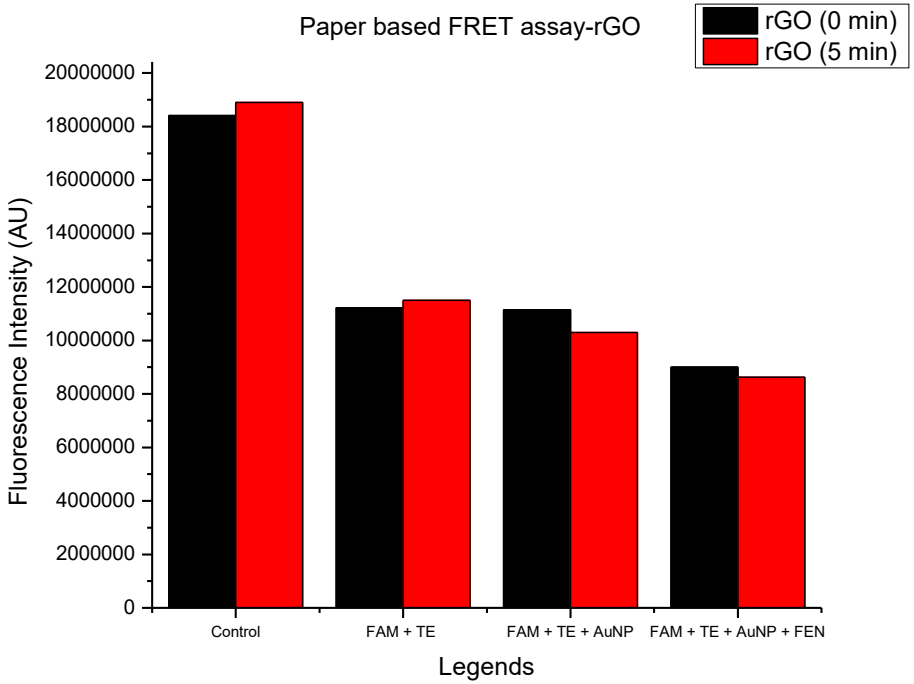



Paper based FRET assay- rGO
r-GO (0min)
r-GO (5min)
Fluorescence Intensity (AU)
50000000
40000000
30000000
20000000
10000000
0
Control
FAM
FAM + AuNP
AuNP + FAM + FENT
Legends

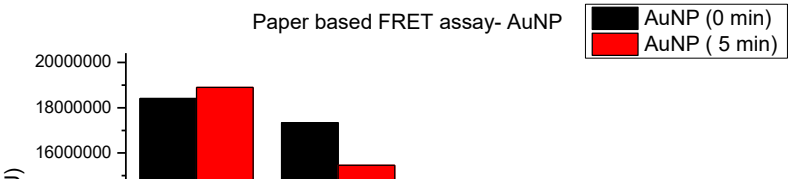
SET 2: Assays with TE buffer:

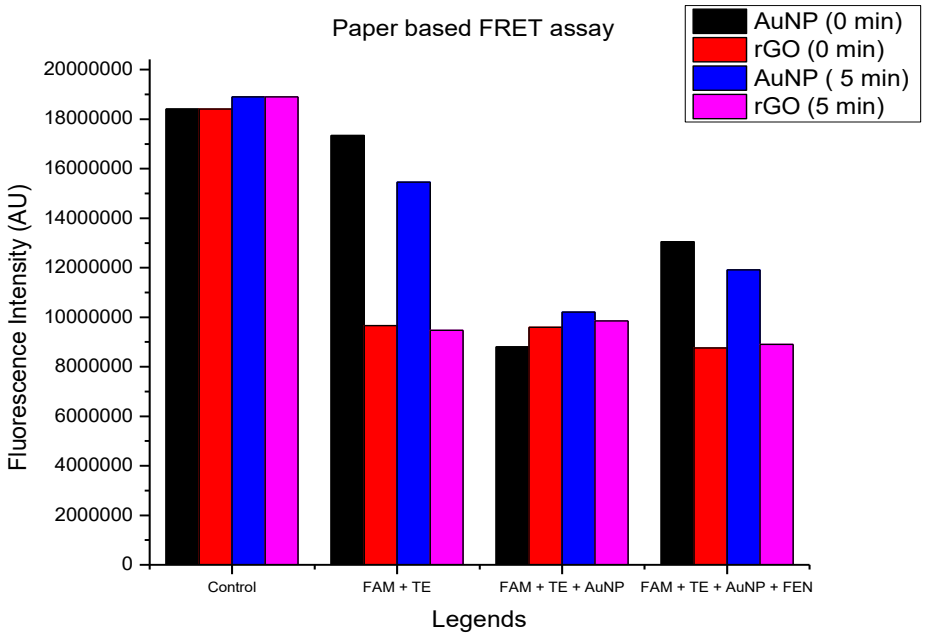


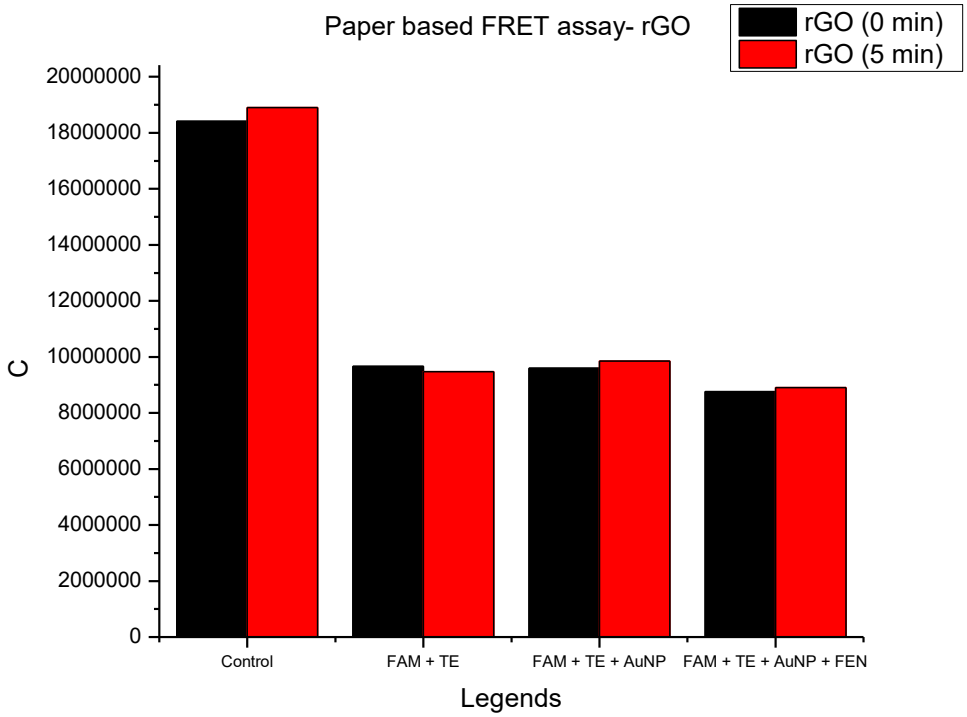




SET 3: Second assay with TE buffer:

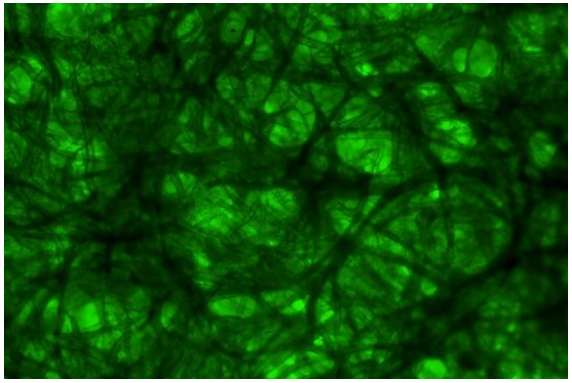



Paper based FRET assay
AuNP (0 min)
rGO (0 min)
AuNP (5 min)
rGO (5 min)
Fluorescence Intensity (AU)
20000000
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
Control
FAM + TE
FAM + TE + AuNP
FAM + TE + AuNP + FEN
Legends

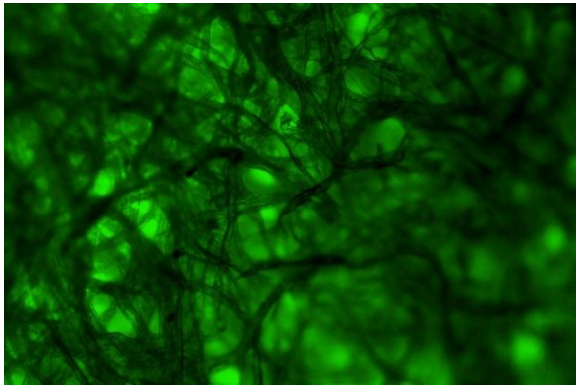

Paper based FRET assay- rGO
rGO (0 min)
rGO (5 min)
20000000
18000000
16000000
14000000
12000000
10000000
8000000
6000000
4000000
2000000
0
C
Control
FAM + TE
FAM + TE + AuNP
FAM + TE + AuNP + FEN
Legends

Fluorescence microscopy results:

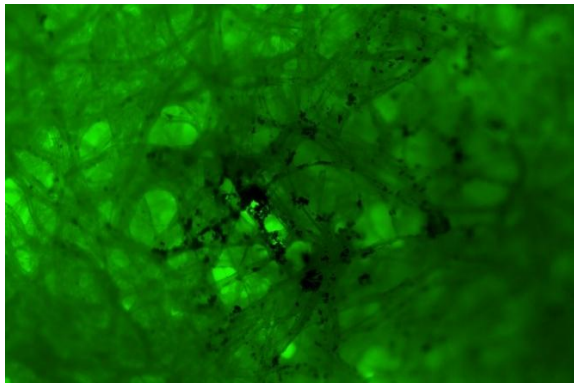
Assay 1- RGO + FAM- labelled aptamer + Fentanyl:



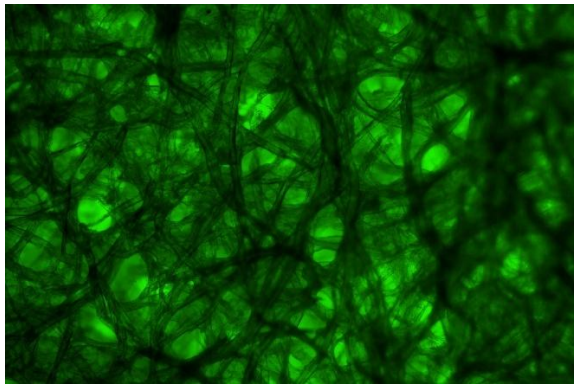
Control



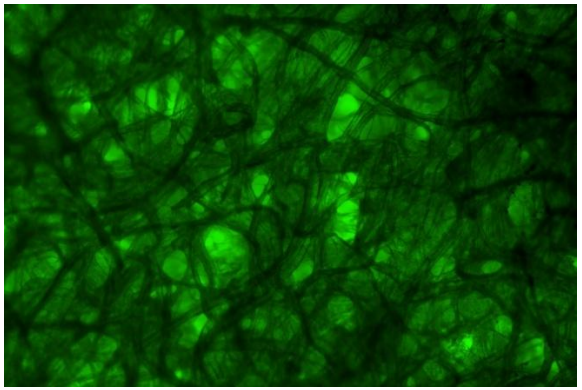
FAM



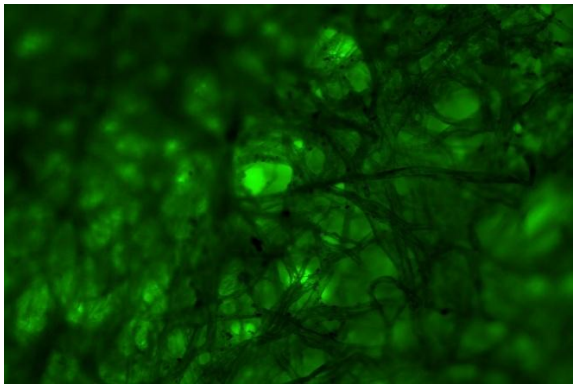
FAM + RGO



FAM + RGO (2)

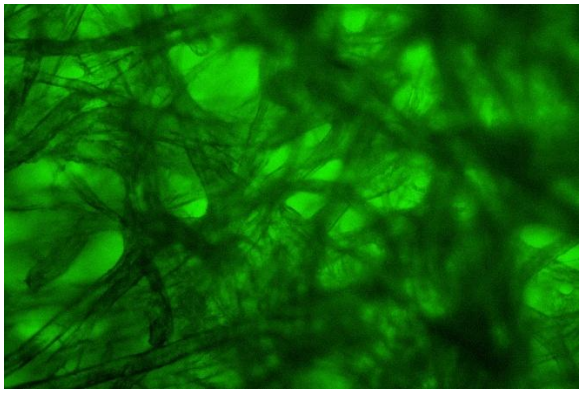


FAM + RGO+ FEN

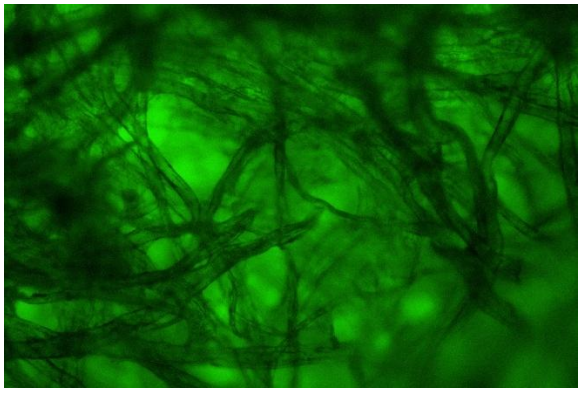


FAM + RGO + FEN (2)

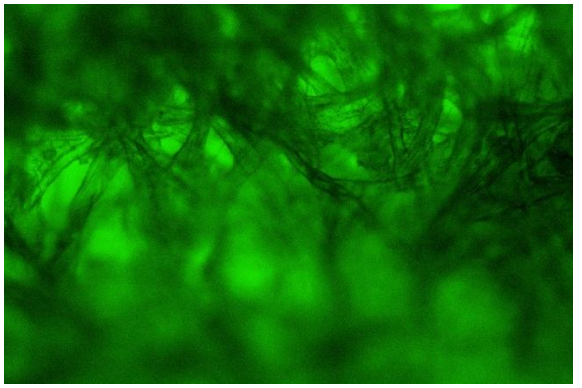
Assay 2- FAM- labelled aptamer + AuNP + FEN



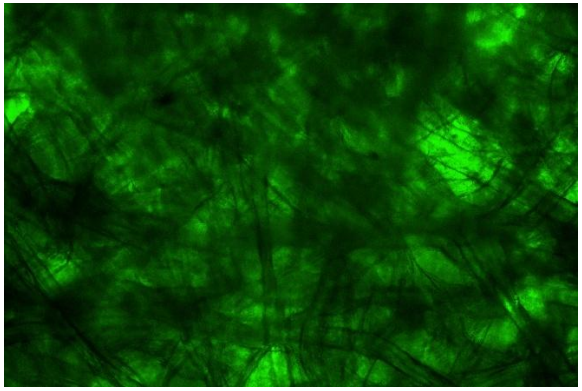
Well A1- FAM



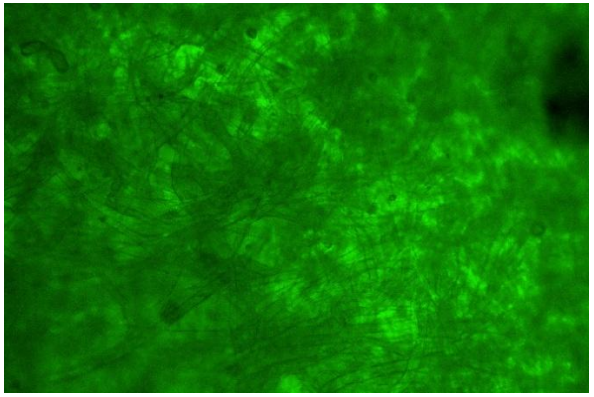
Well A2- AuNP + FAM



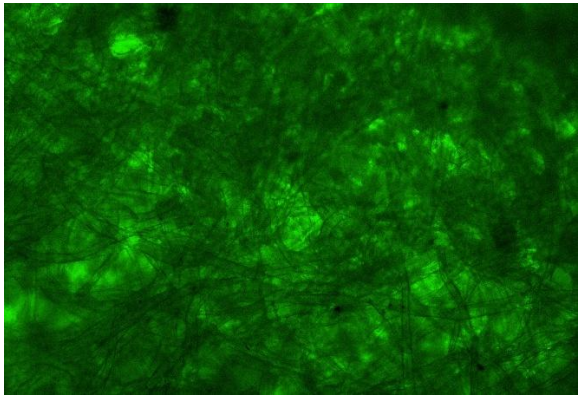
Well A3- FAM + AuNP + FEN



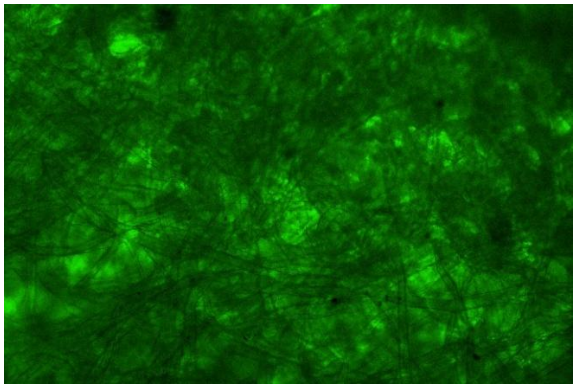
Well A4- FAM + TE



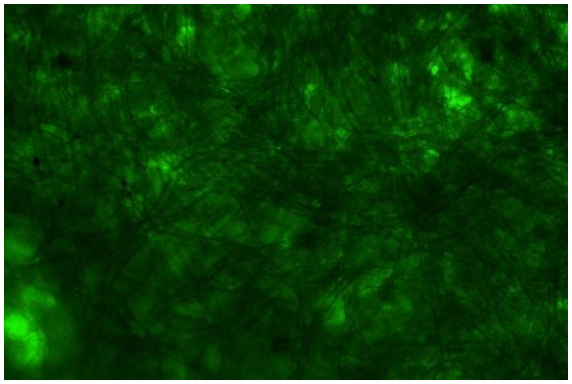
A5- AuNP + FAM + TE



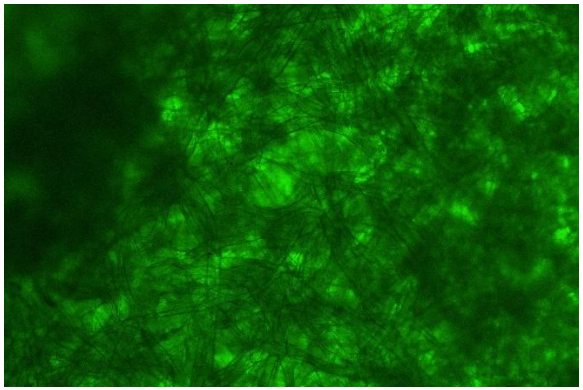
A6- AuNP + FAM + FEN + TE



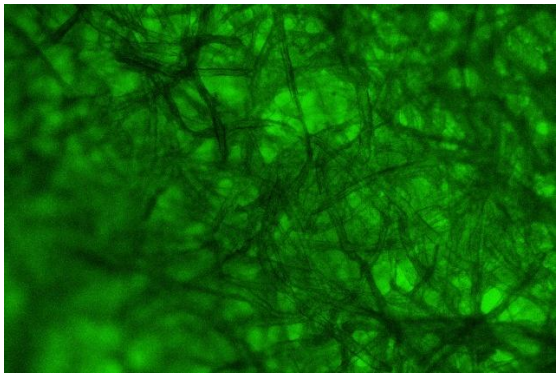
A7- FAM + TE



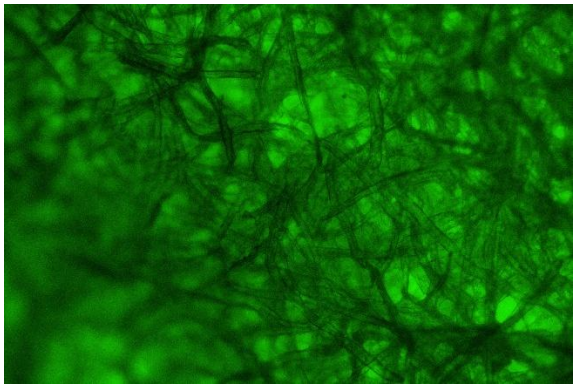
A8- AuNP + TE + FAM



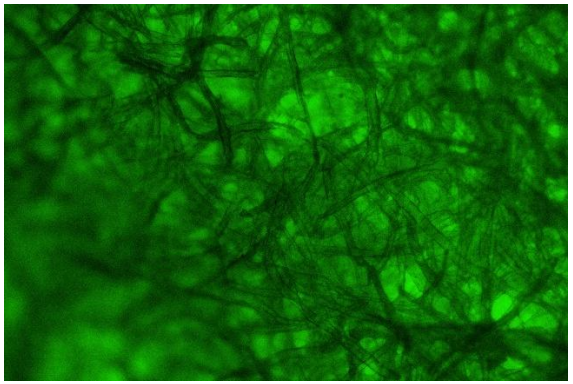
A9- AuNP + TE + FAM + FEN



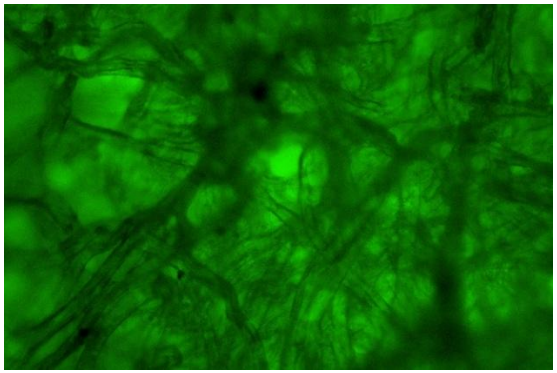
A11- Control



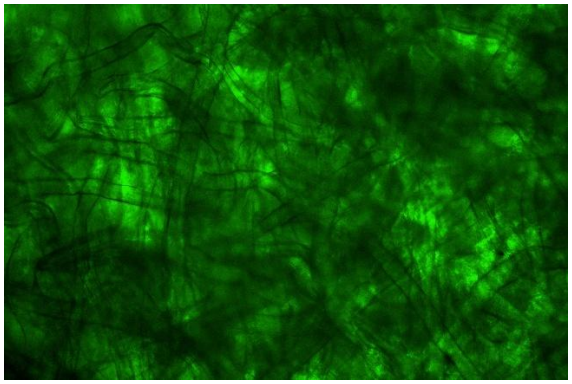
B1- FAM



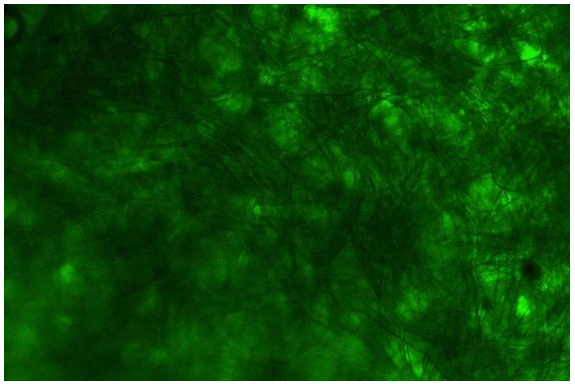
B2- AuNP + FAM



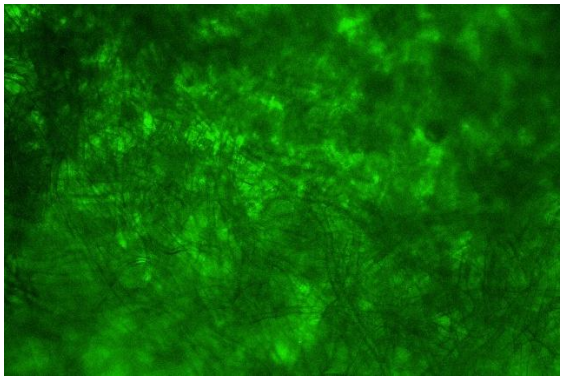
B3- AuNP + FAM + FEN



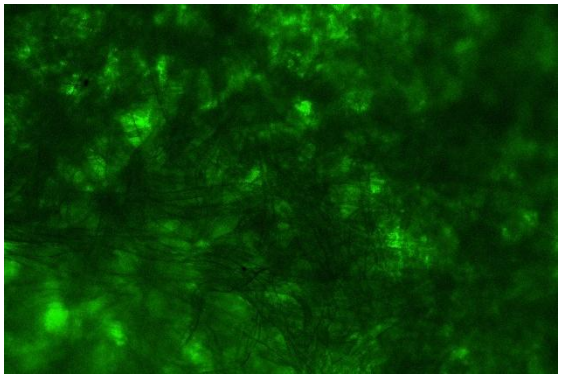
B4- FAM + TE



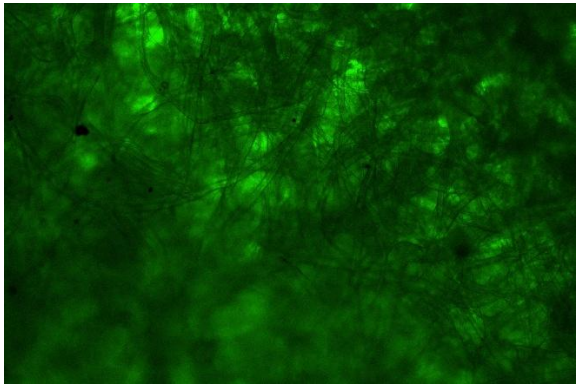
B5- AuNP + FAM +TE



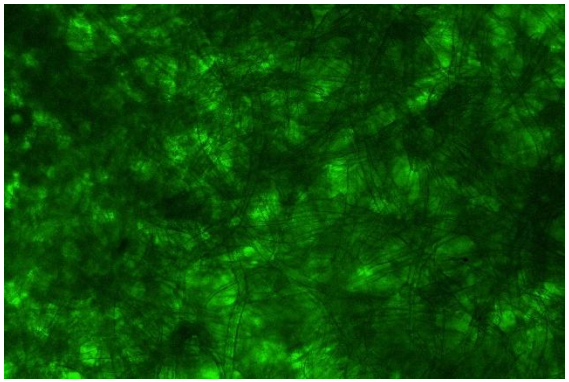
B6- AuNP + FAM



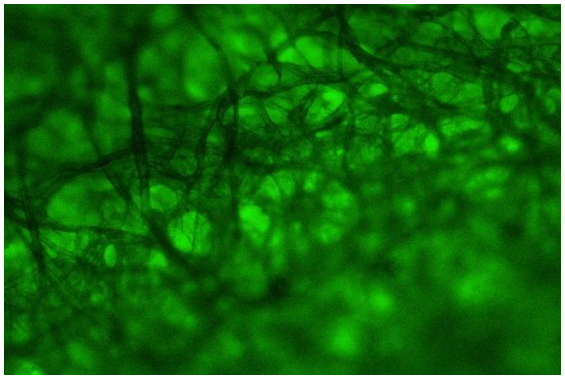
B7- FAM + TE



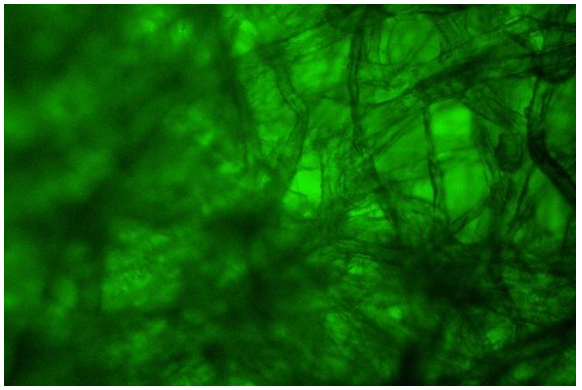
B8- AuNP + FAM + TE



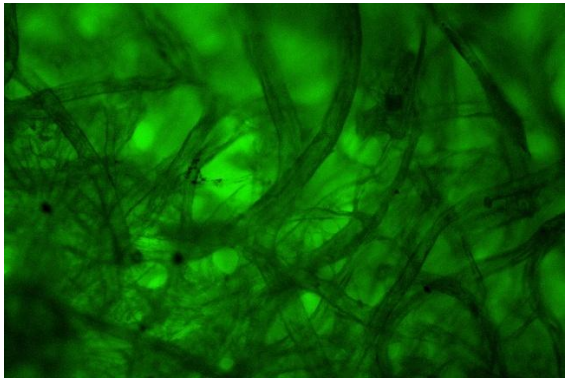
B9- AuNP + TE + FAM



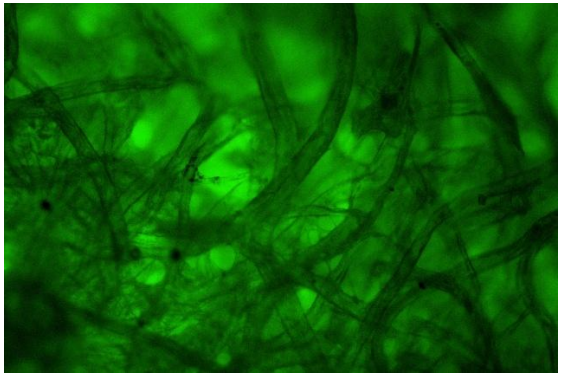
B11- Control



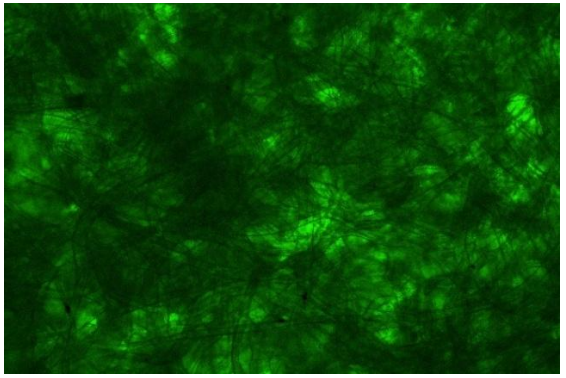
C1- FAM



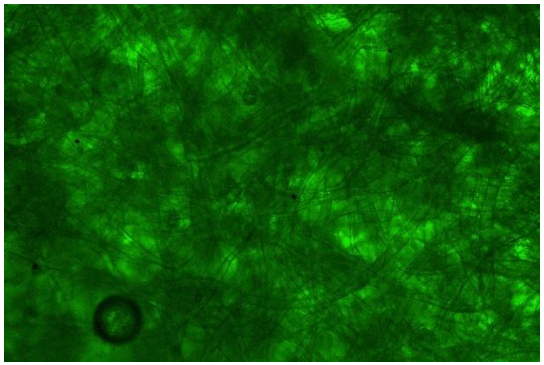
C2- AuNP + FAM



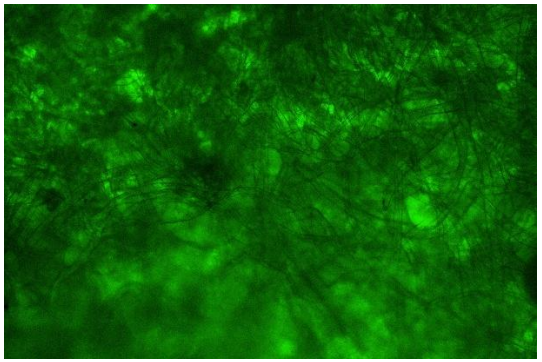
C3- AuNP + FAM + FEN



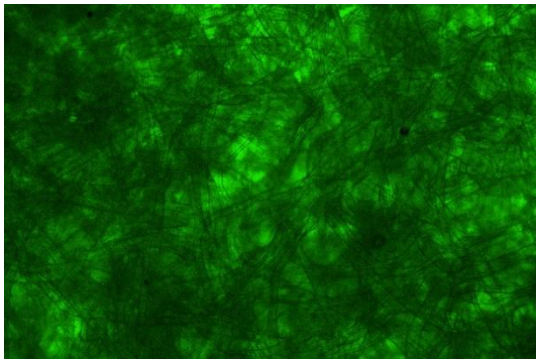
C4- FAM + TE



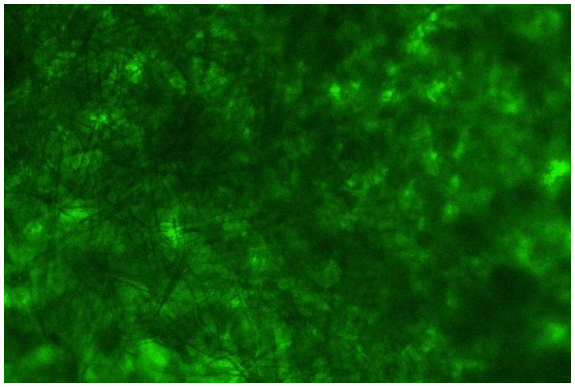
C5- AuNP + FAM + TE



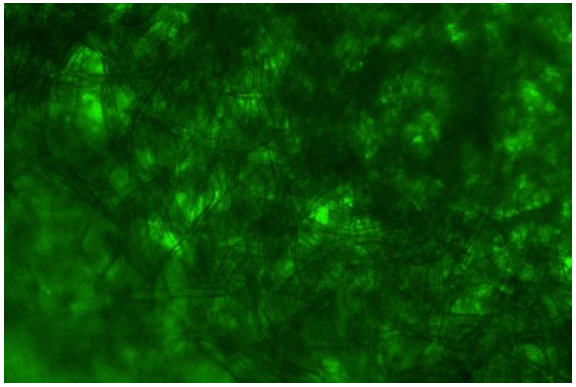
C6- AuNP + FAM + FEN + TE



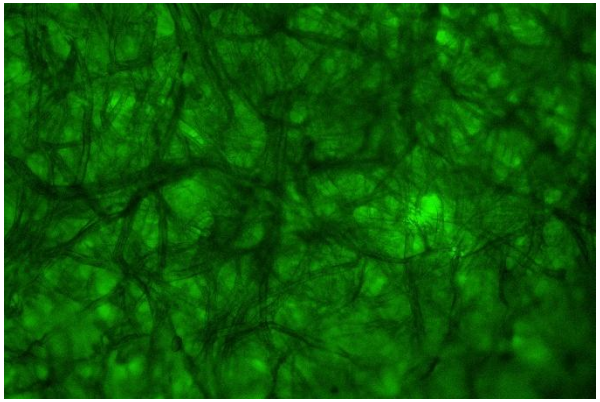
C7- FAM + TE



C8- AuNP + TE + FAM

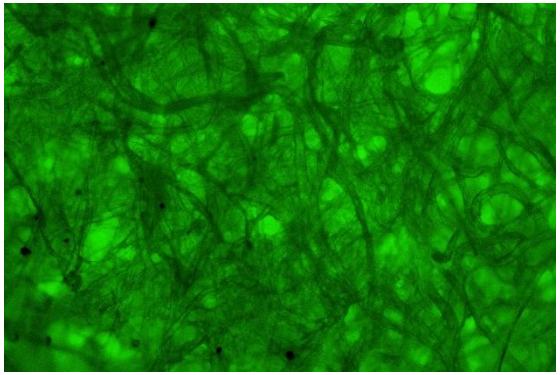


C9- AuNP + TE + FAM + FEN

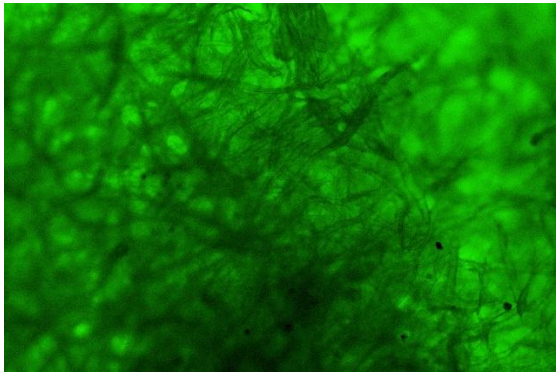


C11- Control

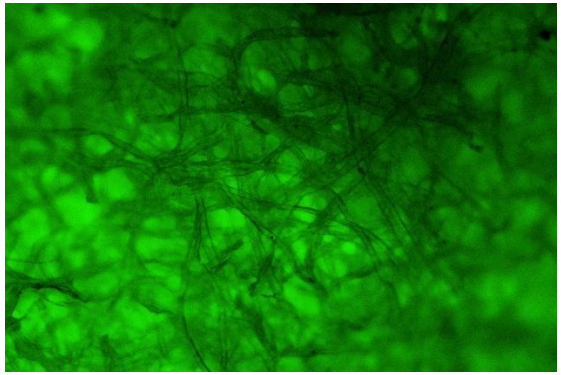
Assay 3- RGO + FAM- Labelled Aptamer + TE + Fentanyl



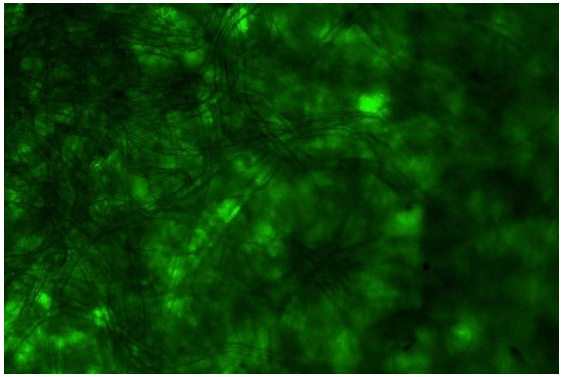
F1- FAM



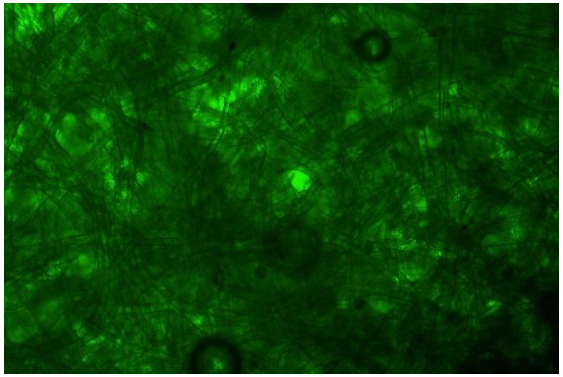
F2 - RGO + FAM



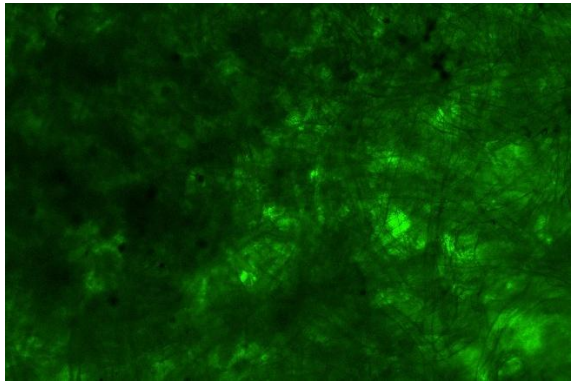
F3 – RGO + FAM + FEN



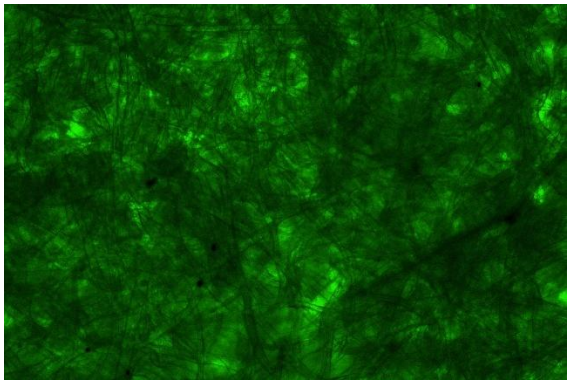
F4 – FAM + TE



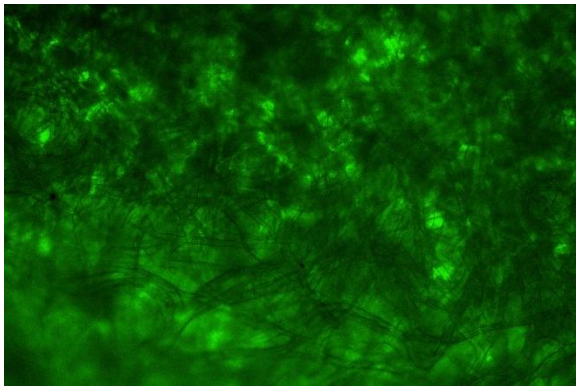
F5 – RGO + FAM + TE



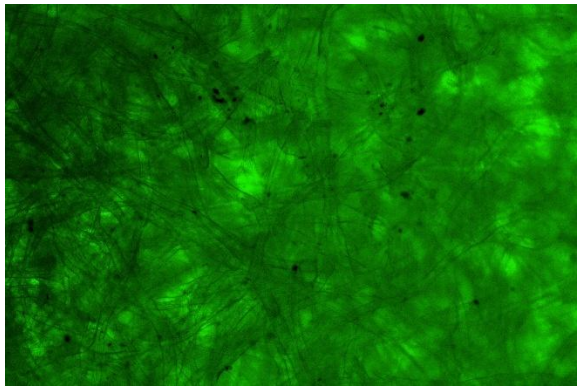
F6 – RGO + FAM + FEN + TE



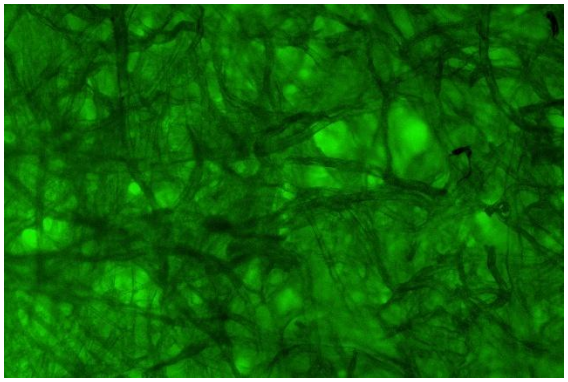
F7 – FAM + TE



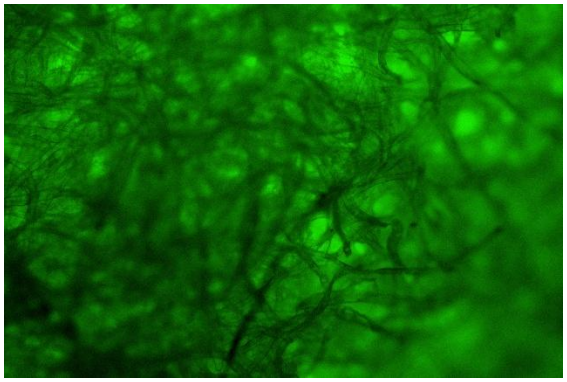
F8 – RGO + TE + FAM



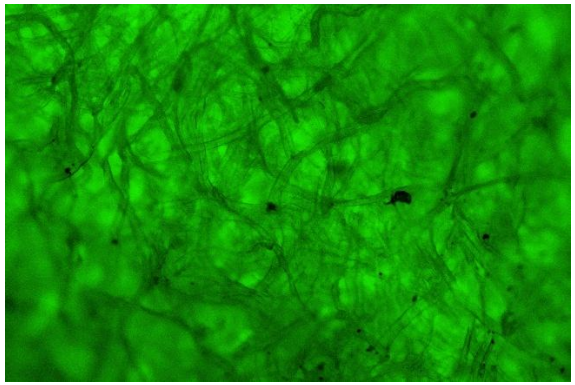
F9 – RGO + TE + FAM + FEN



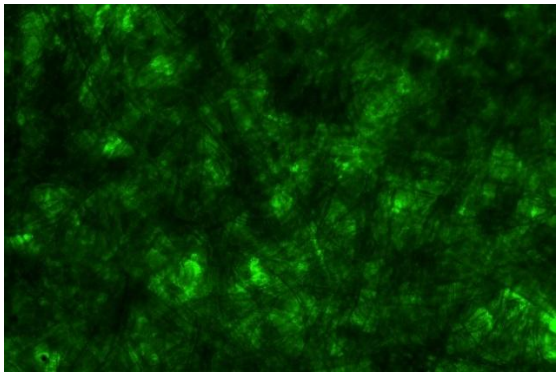
G1 – FAM



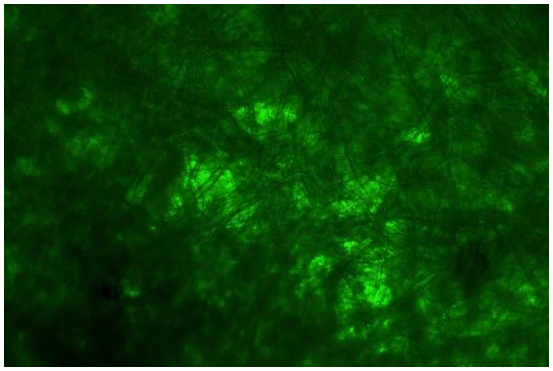
G2 – RGO + FAM



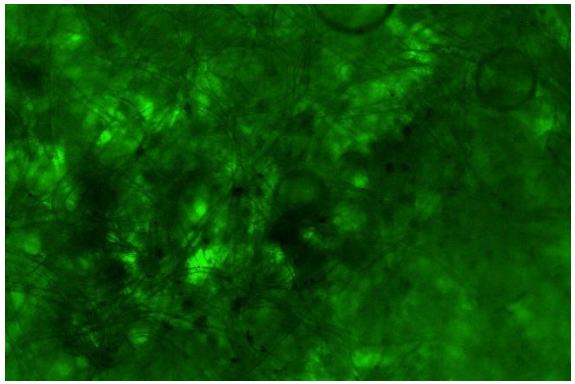
G3 – RGO + FAM + FEN



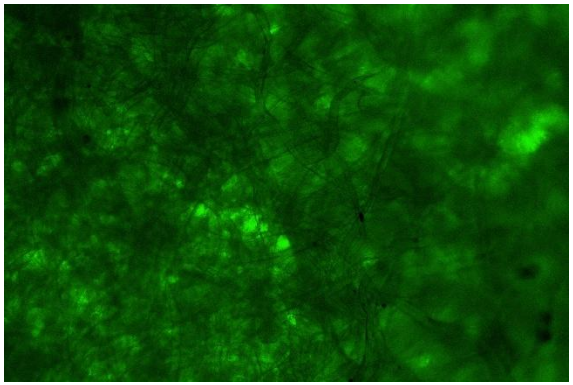
G4 – FAM + TE



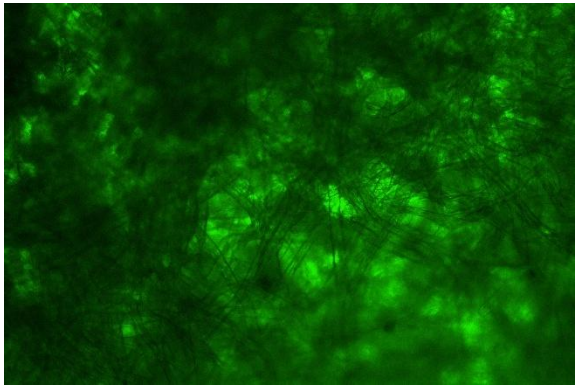
G5 – RGO + FAM + FEN + TE



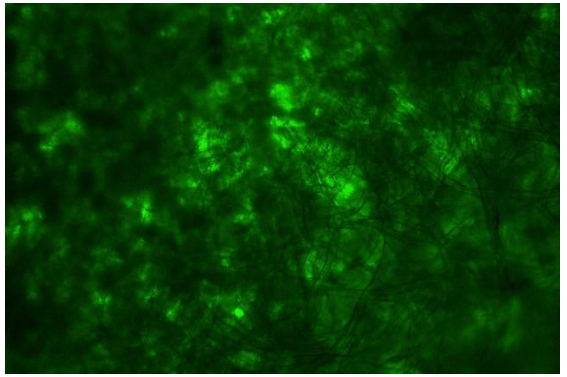
G6 – RGO + FAM + FEN + TE



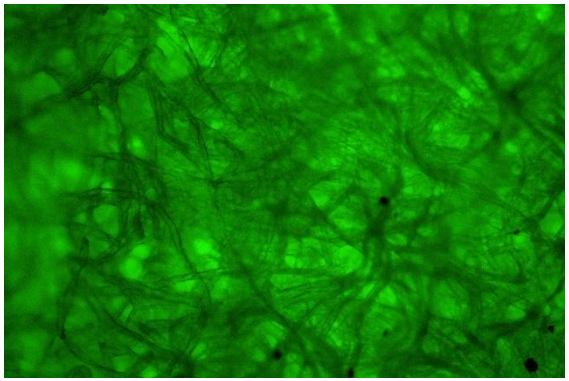
G7 – FAM + TE



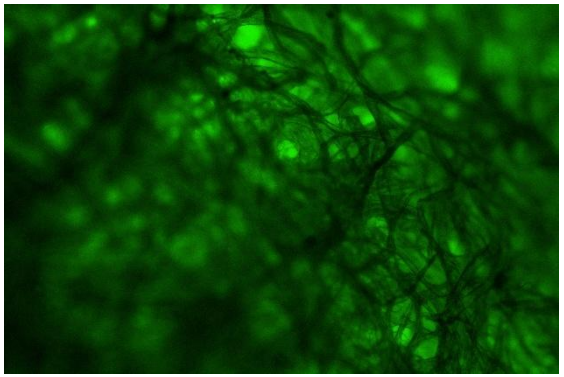
G8 – RGO + TE + FAM



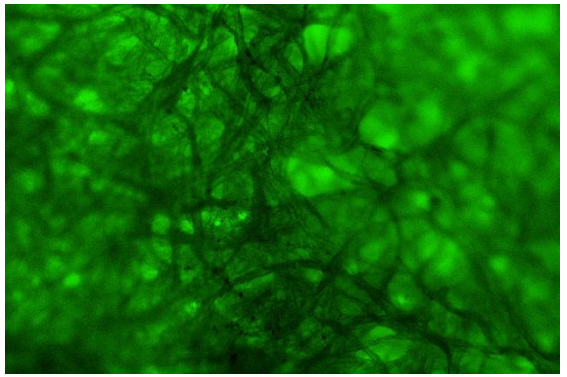
G9 – RGO + TE + FAM + FEN



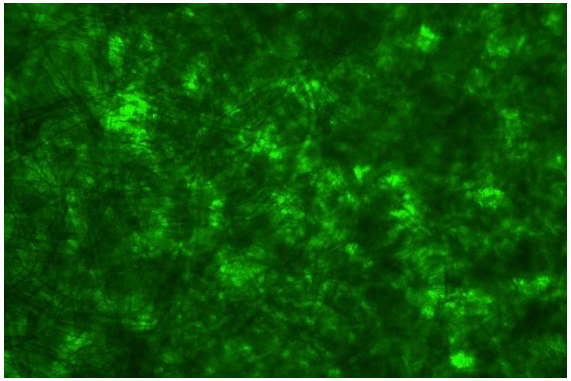
H1 – FAM



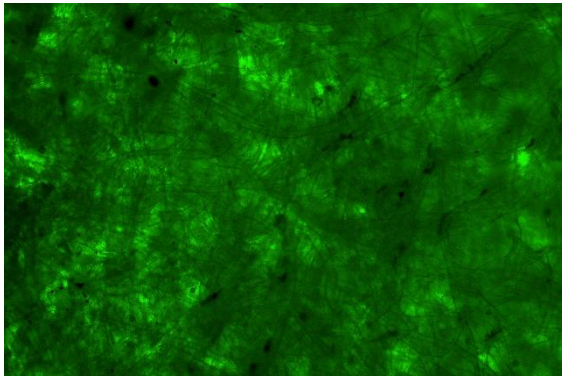
H2 – RGO + FAM



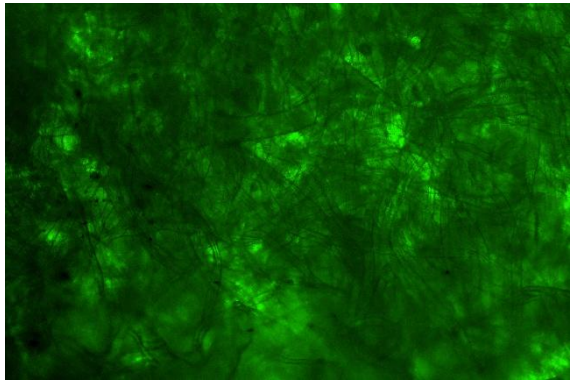
H3 - RGO + FAM + FEN



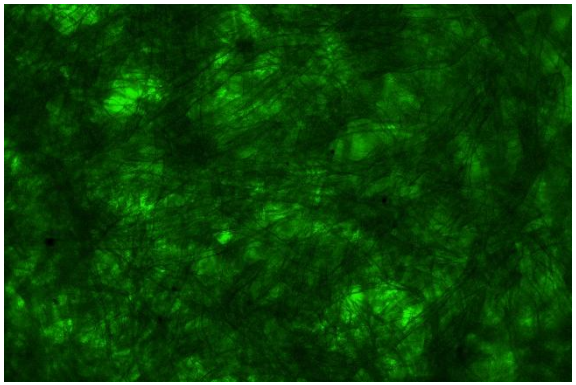
H4 – FAM + TE



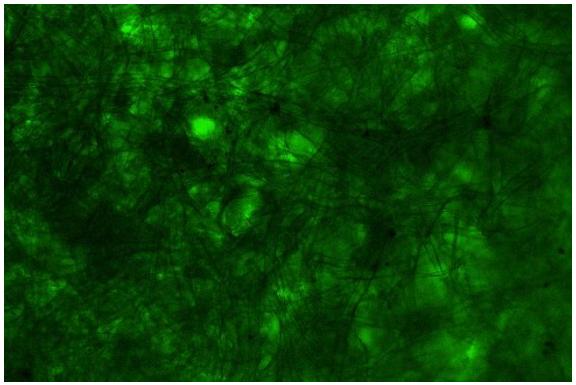
H5 – RGO + FAM + TE



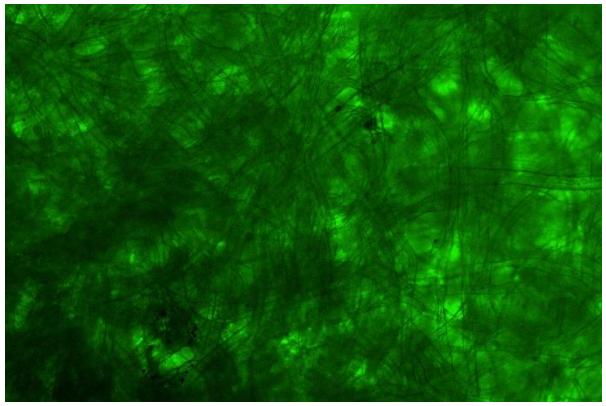
H6 – RGO + FAM + FEN + TE



H7 – FAM + TE



H8 – RGO + TE + FAM



H9 – RGO + TE + FAM + FEN