In situ simultaneous monitoring of ATP and GTP using a graphene oxide nanosheet–based sensing platform in living cells

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Here we present a detailed protocol for *in situ* multiple fluorescence monitoring of adenosine-5′-triphosphate (ATP) and guanosine-5′-triphosphate (GTP) in MCF-7 breast cancer cells by using graphene oxide nanosheet (GO-nS) and DNA/RNA aptamers. FAM-labeled ATP aptamer and Cy5-modified GTP aptamer are used to construct the multiple aptamer/GO-nS sensing platform through 'π-π stacking' between aptamers and GO-nS. Binding of aptamers to GO-nS guarantees the fluorescence resonance energy transfer between fluorophores and GO-nS, resulting in 'fluorescence off'. When the aptamer/GO-nS are transported inside the cells via endocytosis, the conformation of the aptamers will change on interaction with cellular ATP and GTP. On the basis of the fluorescence 'off/on' switching, simultaneous sensing and imaging of ATP and GTP *in vitro* and *in situ* have been realized through fluorescence and confocal microscopy techniques. In this protocol, we describe the synthesis of GO and GO-nS, preparation of aptamer/GO-nS platform, *in vitro* detection of ATP and GTP, and how to use this platform to realize intracellular ATP and GTP imaging in cultured MCF-7 cells. The preparation of GO-nS is anticipated to take 7–14 d, and assays involving microscopy imaging and MCF-7 cells culturing can be performed in 2–3 d.

INTRODUCTION

Simultaneous sensing and imaging of multiple molecules inside living cells is a powerful technique for exploring protein functions in biological systems or for elucidating dynamic biological processes such as metabolic activity, cell proliferation, apoptosis, receptor occupancy, reporter gene expression and antigen modulation[1–3]. Nucleotides have important roles in metabolism and in metabolic control. Among them, ATP and GTP are found as typical energy molecules regulating various biological processes. Accordingly, a suitable *in situ* analyzing platform for simultaneous ATP and GTP visualization in living cells is highly desirable. To this end, a variety of analytical approaches have been developed for highly selective and sensitive detection of ATP and/or GTP molecules[4–9]. However, the development of sensing agents for ATP and/or GTP detection still remains a major challenge in the field of molecular imaging.

Fluorescent probes used for ATP or GTP detection

At present, artificial fluorescent probes based on synthetic chemicals have been used for ATP or GTP detection. These include 2,2′-dipicolylamine-zinc(II), polythiophene derivative, benzimidazolium dyes, sandwich-stacking of pyrene-adenine-pyrene, and so on[10–20]. However, most of these sensing probes are only being used for *in vitro* assays, and their potential for live-cell imaging is highly limited because they are not able to go through the cell membrane. For example, it is difficult to apply the fluorescent polythiophene or trisulfonate derivative to image cellular ATP or GTP despite their selectivity in aqueous solution[17,20]. More recently, promising approaches for ATP and/or GTP detection based on aptamers have been developed. Aptamers are nucleic acids that are isolated from combinatorial oligonucleotide libraries by *in vitro* selection on the basis of their binding to a specific ligand of interest. The target versatility, the high binding affinity and specificity make aptamers fascinating as molecular tools for bioanalytical applications[21–23]. To date, ATP aptamer sensors that involve fluorescent, electrochemical and colorimetric methods have been reported in numerous studies[24,25]. Unfortunately, lack of intracellular transportation for aptamer probes restricts their further applications in cellular sensing *in vivo*.

Graphene oxide nanomaterials

By using the chemically modified graphene oxide nanomaterials, we and others have successfully performed graphene-based biosensing and biochemical analysis[26–35]. Recently, our group has developed a proof-of-concept analyzing assay for cellular ATP detection in living cells[36]. This method has fully used GO-nS as a multifunctional unit as a robust quencher for fluorescent dye, an efficient carrier for aptamer probes and also a strong protector regarding enzymatic cleavage. GO-nS is a chemically modified graphene oxide with a small size (<100 nm) and a narrow size distribution. As a functionalized nanomaterial, GO-nS features (i) efficient cell-membrane permeability, (ii) low toxicity and (iii) sensitive detectability by confocal microscopy. It is reported that nanomaterials are taken up by cells in different ways, including clathrin-mediated endocytosis, caveolae-mediated endocytosis and phagocytosis, which are highly dependent on cell types and physicochemical properties of nanomaterials such as size, charge, elemental composition, surface area and surface chemistry[37]. In the present protocol, the cell-membrane permeability and the aptamer probe transportation of GO-nS are mainly derived from its small size, good dispersing ability in physiological environment and chemical composition.

In addition, GO-nS has shown marked enzymatic cleavage protection for aptamer probe. As we know, most biological probes, such as mRNA and molecular beacons, are easily degraded by

cellular enzymes or digested by cellular nucleases, which limits their applications in living-cell studies. Therefore, delivery of aptamer probes into cells while protecting the fluorescent aptamers from enzymatic cleavage is a key step toward facilitating the biological application of aptamers. To date, only a few nanomaterials (such as carbon nanotubes, silica nanoparticles and gold nanoparticles) have demonstrated protective capabilities during molecular transport. Agarose gel electrophoresis is included in the PROCEDURE (**Box 1**) so that the GO-nS–dependent protection of aptamer from enzymatic cleavage can be confirmed. We have also included the procedure for performing an enzymatic cleavage protection test. It is not necessary to do this routinely for cellular target monitoring or cell imaging, but if the cellular sensing performance is poor or if cell imaging has failed, this is an important quality control step for GO-nS.

GO-nS shows promise as an artificial nanomaterial in cell imaging and *in situ* monitoring, because of its ability to adsorb DNA coupled with its quenching capacity for fluorophores (FAM and Cy5, for example, when labeled to ATP aptamer and GTP aptamer) with a wide energy transfer range. In this protocol, we systematically summarize the preparation and characterization of GO-nS, and report the detailed procedures for applying this GO-nS–based sensing technology to the simultaneous cellular imaging of ATP and/or GTP[38].

Monitoring and imaging multiple analytes

The results so far obtained using the present protocol indicate that GO-nS-based sensing system holds promise for the monitoring and imaging of multiple analytes in living cells. For example, on the basis of the principle of complementary base pairing or aptamer recognition ability, a dye-labeled DNA probe or aptamer probe could interact with the complementary target DNA sequences or target proteins, respectively, in the cells to be studied. With the formation of a DNA double helix (probe DNA/target DNA) or combinatorial structures (aptamer probe/target protein), the fluorescence of quenched dye molecules is recovered with the splitting of ds-DNA or combinatorial aptamer/protein structures from the GO-nS–sensing platform. These designs, with the help of the GO-nS–based sensing platform, will be capable of cellular simultaneous imaging studies of other analytes and biomarkers. One concern that needs to be pointed out here is the necessity of the cell viability assay (**Box 2**). We recommend using the cell viability assay for quality control of GO-nS before cell imaging, especially when using different cell lines. The cell viability assay is not a necessary step for cell experiments if the procedure works well.

Description of the method

To validate the utility of this technology in our study, we used fluorescent dye–labeled DNA/RNA aptamers and GO-nS to create the sensing platform[36,38]. By taking advantage of aptamers and GO-nS, we can load ATP- and GTP-selective aptamer probes onto GO-nS through self-assembly and realize the cellular delivering successfully. Owing to the electron acceptor effect of GO-nS, we can obtain an obvious fluorescent off/on switch and real-time target detection in living cells. In addition, GO-nS shows excellent protection for DNA/RNA from enzymatic cleavage and it shows good biocompatibility with living cells in studies. **Figure 1** shows the workflow of the *in situ* molecular probing in living cells by using aptamer/GO-nS nanocomplexes.

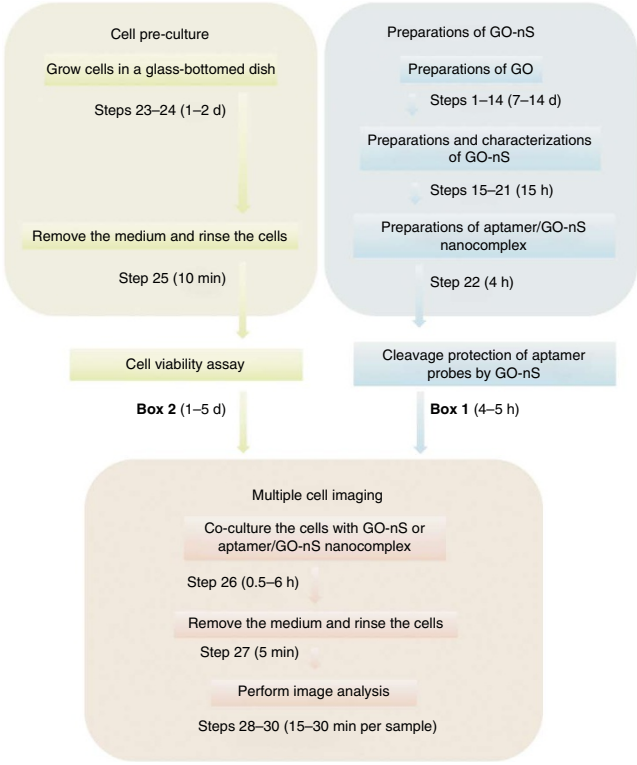

Figure 1 | Workflow of the process of *in situ* molecular probing in living cells by using the aptamer/GO-nS nanocomplex. The procedure comprises the preparation of specimens for analysis, preparation of GO-nS and imaging analysis.

Experimental design

Preparation of GO-nS. GO was synthesized by the Hummers and Hoffman method with some modifications, followed by strong sonication to disperse GO in water and centrifugation to remove large GO layers. It is worth mentioning that commercial graphene or GO materials are available for purchase, which would simplify the protocol; researchers could then skip ahead to 'Preparation of GO-nS' (Steps 15–21). To obtain graphene oxide particles that are of sufficiently small (<100 nm) and uniform size to form acceptable graphene oxide solutions, we use high-power sonication in a strong basic environment at low temperature. To demonstrate that GO-nS is more water-soluble and stable than chemically synthesized graphene oxide, we carried out the characterizations of prepared GO-nS materials and provided the details in supporting information. Detailed information and supporting experiments comparing GO and GO-nS can be found in **Supplementary Figures 1–8** and in our previous study and its supporting information file (ref. 36; http://pubs.acs.org/doi/suppl/10.1021/ja103169v/suppl_file/ja103169v_si_001.pdf). It is important to check the quality of GO-nS on the basis of various characterization approaches, such as the dispersing ability in reaction buffer and cell culture medium with the naked eye, transmission electron microscopy (TEM), X-ray diffraction (XRD), and Raman and Fourier transform IR (FT-IR) spectroscopy.

Configuration of the DNA/RNA aptamer/GO-nS sensing platform. Figure 2 shows the basic concept of the proposed sensing platform. Aptamers are short, single-stranded oligonucleotides that are selected by an *in vitro* method known as SELEX (systematic evolution of ligands by exponential enrichment).

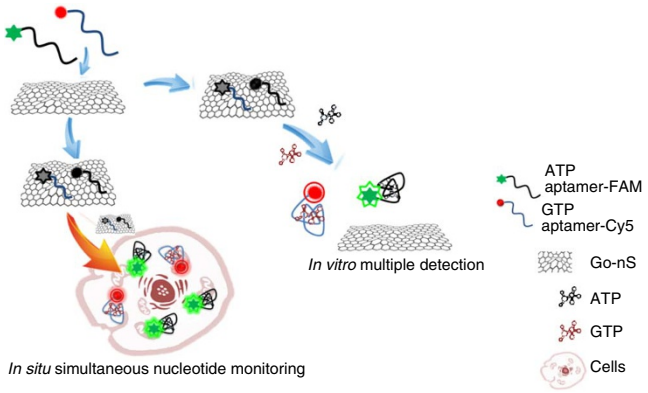

Figure 2 | Schematic illustration of *in vitro* and *in situ* molecular probing in living cells by using the aptamer/GO-nS nanocomplex. Binding of ATP aptamer–FAM and GTP aptamer–Cy5 to GO-nS leads to fluorescence 'off' owing to the FRET between fluorophores and GO-nS. After incorporating the analytes (ATP or GTP), loop-structured assemblies of aptamer-ATP and aptamer-GTP are released from GO-nS and result in fluorescence 'on'. *In situ* simultaneous probing of ATP and GTP in living cells can consequently be done by using this fluorescence off/on switch concept.

At present, aptamers have been used in many applications ranging from fundamental studies to therapeutic research[39,40]. The first RNA aptamer with high specificity for the GTP molecule was reported by Szostak and co-workers in 2002 (refs. 41–45). This RNA aptamer is able to target GTP by forming a loop structure with K_d values ranging from 9 nM to 8 μM. Here we design the multiple sensing platform by loading the DNA/RNA aptamers on GO-nS.

GO-nS has a large surface-to-volume ratio, and the planar structure provides a suitable substrate for assembling multiple probes. By using carboxyfluorescein (FAM)-labeled ATP aptamer and cyanine-5 (Cy5)-modified GTP aptamers, we can achieve the multiple targeting of ATP and GTP simultaneously. Aptamers are strongly anchored onto the GO-nS surface through 'π-π stacking'[34,36,38]. As shown in **Figure 2**, binding of aptamers to GO-nS guarantees the close proximity of dyes to the graphene surface. The subsequent efficient long-range energy transfer from dye to GO-nS results in rapid and complete fluorophore quenching. In direct contrast, after interaction with ATP or GTP, the aptamers will change into stable and internal loop structures. The weak binding ability of the loop-structured assembly of the aptamer/target to GO-nS keeps fluorophores far away from the quencher surface, leading to fluorescence recovery of FAM or Cy5. As a result, fluorescence signals will appear again when the emission scan is acquired.

***In situ* molecular probing in living cells by using an aptamer/GO-nS nanocomplex.** By referring to previous studies of drug delivery and tumor diagnostics using graphene derivates, we believe that GO-nS is a suitable tool for visualization of multiple nucleotides based on the fluorescent off/on switch mechanism. This can be demonstrated by incubating MCF-7 cells (a human breast cancer cell line) with aptamer/GO-nS complex consisting of ATP aptamer–FAM, GTP aptamer–Cy5 and GO-nS. We use a confocal microscope to capture the cell-imaging photos (**Fig. 2**). Fortunately, the pictures show bright fluorescent signals corresponding to FAM and Cy5 tagged on DNA or RNA aptamers released from the complex after interaction with cellular adenine derivates (including ATP, AMP and adenine) and guanosine derivates. In this protocol, we test the total amount of adenine derivates (including ATP, AMP and adenine) and guanosine derivates in living cells.

Advantages and limitations of this protocol to investigate *in situ* cellular nucleotides. There are a number of protocols available to investigate the live-cell imaging probes (see refs. 46–49 for instance). Here we only focus on the advantages and limitations of the present imaging method in comparison with some other methods reported previously for ATP or GTP detections and imaging (**Table 1**). We also discuss the advantages and specificity of this protocol for studying molecular probing in living cells *in situ*:

(i) GO-nS is a 2D nanomaterial with a large surface/volume ratio. The GO-nS surfaces are considered as active surfaces for the loading of aptamer probes, as well as for immobilization. As a result, we could achieve multiple loading of aptamer probes with higher efficiency by using GO-nS in this protocol.
(ii) Charge carriers in graphene obey a linear dispersion relation near the Fermi energy and behave as massless Dirac fermions, as observed in the quantum Hall effect and the ambipolar electric field effect. Because of these unique properties, GO-nS exhibits super-quenching ability during the FRET process. The high quenching efficiency makes GO-nS a sensitive sensing platform for fluorescence detection and monitoring of the target species.
(iii) Generally, we use commercial transfection agents such as Lipofectamine to transfect either plasmids or DNA or RNA in a general molecular biology laboratory. However, such a lipid agent can only act as a carrier and realize the transportation of DNA or RNA through the cell membrane. In the protocol that we report, GO-nS not only enables transportation but also acts as a sensing platform with high fluorescence quenching efficiency and provides protection of the probe DNA against enzyme cleavage.

Although this protocol is aimed at introducing a universal living-cell sensing platform, it is worth mentioning that several concerns need to be pointed out and there may be a need to modify this protocol for specific studies. First, we have tested two different cell types, including mouse epithelial cells (JB6 Cl 41-5a) and human breast adenocarcinoma cells (MCF-7 cells, (ATCC,HTB-22)), using our probes, wherein the cell imaging procedure conditions such as working concentrations and incubation times are determined. Therefore, it is possible that for other cell types this procedure may not be optimal and need modification. Second, although toxicity is minimal at working concentration, the probes show some signs of toxicity at higher concentrations, especially after long cell culture time (>70 h). If the readers need to carry out long-term online monitoring, additional studies would be essential to establish a new working procedure. The third limitation concerns the synthesis and preparation of GO-nS, as well as the aptamer/GO-nS nanocomplex. Sufficient characterizations such as by TEM, XRD, Raman spectroscopy and so on should be carried out to confirm that the material is up to grade. Still, control

TABLE 1 | Advantages and limitations of different methods for ATP and/or GTP detection and imaging.

Tool	Application	Pros	Cons	Refs.
Luciferase bioluminescence assay	Direct detection of ATP	Sensitive with high affinity, differentiable for cytoplasm ATP and mitochondria ATP, suitable for cell imaging	Requires transfection and bioluminescence; relies on the concentration of luciferase, oxygen and luciferin	5,8
Synthesized fluorescent dyes	Direct detection of ATP	With unique specificity, does not require transfection	Need cell lysis to perform the cellular detection of ATP in buffer solutions	9–13,19,20
Target-responsive Aptasensors	For ATP detection with fluorescent, electrochemical or colorimetric methods	High sensitivity, low price and programmable	Need cell lysis to perform the cellular detection of ATP in buffer solutions	23–25
Organic fluorescent probes	Directed GTP detection	Combine fluorescent dye molecules with designed receptors for GTP	Require organic synthesis, cannot be used for imaging in living cells	14,16,17
Glass bead microarray	For simultaneous ATP and GTP detection in aqueous solutions	Simultaneous assay of ATP and GTP, sensitive to structurally similar molecules	Cannot be used for live-cell imaging	15
GO-nS nanocomplex platform	For simultaneous ATP and GTP detection in living cells	Highly sensitive and specific for structurally similar molecules, does not require transfection, suitable for visualization study	Long-term biosecurity is uncertain for live cell studies, since the biosecurity of nanomaterials has not been demonstrated yet, especially if the protocol is used for *in vivo* experiments; the present protocol cannot be used for quantitative detection of cellular ATP and GTP	This paper; ref. 38

of the size of GO-nS, as well as the size distribution, might be one of the foremost challenges for the protocol. For this reason, we limited our application of this protocol to ATP and GTP so far; modifications to this procedure might yet be necessary to satisfy the unique criteria of specific molecular profiling applications such as microRNAs and mRNAs.

MATERIALS

REAGENTS

! CAUTION All chemicals and reactions used in this protocol are potentially harmful, and thus a lab coat, gloves and eye protection should be used.

- Double-distilled water (from a Barnstead NANO Pure UV ultrapure water system)
- Graphite powder (<45 μm, ≥99.99%; Sigma-Aldrich, cat. no. 496596-113.4G)
- Sulfuric acid (H_2SO_4; Sigma-Aldrich, cat. no. 339741-100ML)
  ! CAUTION Sulfuric acid is highly corrosive.
- Potassium peroxodisulfate ($K_2S_2O_8$; Sigma-Aldrich, cat. no. P5592-500G)
- Phosphorus pentoxide (P_2O_5; Sigma-Aldrich, cat. no. 431419-50G)
- Potassium permanganate ($KMnO_4$; Sigma-Aldrich, cat. no. 399124-25G)
- Hydrogen peroxide solution (H_2O_2; 35 wt% solution in H_2O, J&K Scientific, cat. no. 916404) **! CAUTION** Hydrogen peroxide solution is highly toxic.
- Hydrochloric acid (HCl; Sigma-Aldrich, cat. no. 84415-100ML)
  ! CAUTION HCl is highly volatile.
- Sodium hydroxide (NaOH; Sigma-Aldrich, cat. no. S8045-500G)
- Magnesium chloride ($MgCl_2$; Sigma-Aldrich, cat. no. 208337-100G)
- Potassium chloride (KCl; Sigma-Aldrich, cat. no. P5405-250G)
- EDTA disodium salt, dihydrate (Sigma-Aldrich, cat. no. E9884-100G)
- ATP disodium salt hydrate (Sigma-Aldrich, cat. no. A26209-1G)
- GTP disodium salt hydrate (Sigma-Aldrich, cat. no. G8877-25MG)
- Cytidine 5′-triphosphate disodium salt (CTP; Sigma-Aldrich, cat. no. C1506-10MG)
- Thymidine 5′-triphosphate sodium salt (TTP; Sigma-Aldrich, cat. no. T0251-10MG)
- Trypan blue (Cellgro, cat. no. 25-900-CI)
- Agarose (Sigma-Aldrich, cat. no. A9414-10G)
- Trypsin-EDTA (0.25% (wt/vol) solution; GIBCO, cat. no. 25300-054)
- FBS (Invitrogen, cat. no. 10099133)
- DNase I (Amp Grade 100 U; Invitrogen, cat. no. 18068-015)
- SYBR Green nucleic acid gel stain starter kit (Invitrogen, cat. no. S-7580)
- 10-bp DNA ladder (Invitrogen, cat. no. 10821-015)
- TrackIt cyan/orange loading buffer (Invitrogen, cat. no. 10482-028)
- PBS buffer (10× concentrate; Sigma-Aldrich, cat. no. P5493-1L)
- TBE buffer (Ambion 10× TBE solution, 44.5 mM Tris-borate and 1 mM EDTA (pH 8.3); Invitrogen, cat. no. AM9863)
- Fluorophore carboxy fluorescein–labeled ATP aptamer (5′-FAM/AACCTGGGGGAGTATTGCGGAGGAAGGT-3′, ordered from Integrated DNA Technologies)
- Cy5-labeled GTP aptamer (5′-Cy5/GGGACGAAGUGGUUGGGUGUGAAAACGUCCC-3′, ordered from Integrated DNA Technologies)
- Alexa Fluor 546–labeled random DNA (Alex546N; 5′-AF546/TCTAAATCGCTATGGTCGC-3′, ordered from Integrated DNA Technologies)
- MEM culture medium with Earle's salts and L-glutamine (GIBCO, cat. no. 11095-080)
- MCF-7 cells (ATCC, HTB-22) **! CAUTION** Human cell cultures are biohazardous and potentially infectious materials. Human cell cultures must be handled in biosafety level 2 (BSL-2) facilities by trained certified personnel. Proper personal protective equipment should be used while handling human cell cultures. Refer to local biosafety regulations for specific requirements.

EQUIPMENT

- Glass beaker, 100 ml, 500 ml and 1 liter

- Round-bottomed flask, 100 ml
- Allihn condenser, 500 mm
- Stirring bar with magnetic core, polytetrafluoroethylene-coated (Fisher Scientific)
- Büchner funnel, 142 mm
- Filter flask, 500 ml
- Nylon film (142 mm, 0.2 µm; Pall, cat. no. 66604)
- Membrane dialysis tubing
- Hot plate magnetic stirrer with contact thermometer oil bath (Fisher Scientific)
- Vacuum pump (Gast)
- Glass-bottom six-well clear plates (Corning, cat. no. 3335)
- Centrifuge tubes (1.5 ml; Eppendorf, cat. no. 31810)
- Clear-bottom dishes, 96 wells, for fluorescent assays (Corning, cat. no. 3583)
- Glass-bottom clear cell culture dishes, 20 mm diameter, for confocal imaging (Nest, cat. no. 801001)
- Microcentrifuge (Thermo Scientific)
- Eppendorf pipettes
- NANO Pure UV ultrapure water system (Barnstead, model no. D11911)
- Atomic force microscope (with Nanoscope III, Veeco)
- Transmission electron microscope (JEOL TEM 2010)
- X-ray powder diffraction meter (D8-Advance, Bruker)
- Raman microprobe system (Renishaw, RM200)
- FT-IR spectrometer (Perkin Elmer)
- Enduro 7.7 horizontal gel box (Labnet, model no. E1007-7)
- CO_2 incubator (Thermo Scientific)
- NucleoVision imaging system with UV irradiation (NucleoTech)
- Laser-scanning confocal microscope (Zeiss, LSM 710 NLO)

REAGENT SETUP

Binding buffer for ATP and GTP assay To 200 ml of binding buffer, add 180 ml of water and 20 ml of 10× PBS directly into a glass bottle with 2.984 g of KCl, 95.3 mg of $MgCl_2$ and 7.45 mg of EDTA. Allow the powder to completely dissolve in PBS. This solution can be capped and stored at 4 °C for 2 weeks. ▲ **CRITICAL** ATP, GTP, TTP and DTP solutions should be prepared with binding buffer (not water) to maintain the concentration of binding buffer during the binding reaction between aptamers and nucleotides.

TBE buffer for electrophoresis To 500 ml of TBE buffer, add 450 ml of water and 50 ml of 10× TBE directly into a glass bottle with 0.372 g of EDTA. Allow the powder to completely dissolve in TBE. This solution can be capped and stored at 4 °C for 2 weeks.

Cells for imaging MCF-7 cells should be cultured in a glass–bottomed microscope dish at 37 °C in a humidified 5% CO_2 atmosphere such that they are ~50–80% confluent on the day of imaging. Complete cell culture medium is combined with complete MEM medium, 0.01 mg/ml bovine insulin and 10% (wt/vol) FBS. For live-cell imaging experiments, seed 6×10^4 cells in 500 µl of complete MEM medium into each glass–bottomed microscope dish, and incubate the dishes at 37 °C for 36 h to ensure that each well has reached ~70% confluency before adding the aptamer/GO-nS complex.

Washing buffer for confocal imaging To 200 ml of washing buffer in a glass bottle, add 180 ml of water and 20 ml of 10× PBS. ▲ **CRITICAL** Washing buffer is used to replace the cell culture medium during confocal imaging. At this point, washing buffer should be prewarmed to 37 °C.

EQUIPMENT SETUP

Confocal microscope Our Zeiss LSM 710 NLO laser-scanning confocal microscope is equipped with an upright Zeiss Axioexaminer stand. The objective is a W Plan-Apo 20× with a numerical aperture (NA) of 1.0 and a water-dipping objective. The laser excitation wavelength for ATP aptamer–FAM is 488 nm (green), for GTP aptamer–Cy5 it is 633 nm (red) and for random DNA sequence–Alex546N it is 561 nm (orange).

PROCEDURE

Synthesis of graphene oxide ● TIMING 7–14 d

1| To a 250-ml round-bottomed flask, add 2 g of graphite powder, 3.0 g of $K_2S_2O_8$, 3.0 g of P_2O_5, and then 12 ml of concentrated H_2SO_4.

2| Turn on the hot plate with a magnetic stirrer, and then set the oil-bath temperature to 80 °C.

3| Fit this round-bottomed flask to a condenser circulating with cold water, turn on the magnetic stirrer and heat the solution at reflux for 4.5 h.

! CAUTION The experiment should be handled carefully in a ventilated fume hood.

4| Allow the flask to cool down to room temperature (25 °C), and then dilute the reaction solution with 500 ml of water.

■ **PAUSE POINT** The reaction solution can be allowed to cool for up to 12 h, or the solution can be stirred overnight (up to 12 h).

5| Remove the solvent by vacuum filtration using a Büchner funnel with a 0.2-µm Nylon film. Wash the solid products three times with water and dry them naturally.

6| Place a 500-ml round-bottomed flask in an ice bath. Keep the temperature at ~0 °C. Put the solid products into a 500-ml round-bottomed flask and add 150 ml of H_2SO_4.

! CAUTION H_2SO_4 is a strong oxidizing agent, and it should be precooled to 0 °C before use.

7| Add 25 g of $KMnO_4$ gradually into the reaction solution. Mix and stir the solution at 35 °C for 2 h.

8| Dilute the reaction solution with 250 ml of water, and keep the water-bath temperature under 50 °C.

! CAUTION H_2SO_4 is a strong oxidizing agent, and the reaction is vigorously exothermic.

▲ **CRITICAL STEP** The H_2O_2 should be added very slowly in a drop-by-drop manner.

9| Transfer the reaction solution into a 2-liter glass baker, add 1 liter of water gradually, and then add 30 ml of 30% (vol/vol) H_2O_2 drop by drop.
▲ **CRITICAL STEP** The H_2O_2 should be added drop by drop. Keep observing the mixture. The color of the solution is supposed to be light brown, and the reaction is supposed to produce vast bubbles.

10| Remove the solvent by vacuum filtration using a Büchner funnel with a 0.2-μm Nylon film. Wash the solid products three times with 1 liter of 10% (vol/vol) HCl and 1 liter of water several times, respectively. Dry the solid products naturally.

11| Prepare 0.5% (wt/vol) graphite oxide solution with water, and remove the free iron or oxidizing molecules from the solution by dialysis.
■ **PAUSE POINT** Transfer the sample into dialysis tubing, tightly seal it and put it into a 2-liter beaker containing 1 liter of water. The dialysis bag should be sealed tightly to avoid leakage of the sample. The dialysis could last for 2 weeks.

12| Remove the solvent by vacuum filtration and dry it using a vacuum pump at or lower than 30 °C for 6 h.

13| Prepare 0.1 mg/ml graphite oxide dispersion with water and then sonicate it for 40 min to ensure exfoliation.

14| Remove the solvent by vacuum filtration, collect the resulting brown precipitate and dry it under vacuum overnight at 25 °C.
■ **PAUSE POINT** At this point, the solid can be stored in the dryer for at least 2 months.

Preparation of GO-nS ● TIMING 7 h
15| Prepare a 0.2 mg/ml GO solution with water, and then sonicate it in a water bath for 2 h.

16| Transfer the solution into an ice bath, and turn on a strong sonification with power of 40 W (4 min/sonication × 5 sonications). The ice bath should be changed after each treatment to keep the sample temperature below 5 °C.
? TROUBLESHOOTING

17| Add NaOH solution (14 mol per liter NaOH solution) to the resultant solution, to obtain a final concentration of 5M NaOH, and then sonicate it in a water bath for 2 h (sonicating power is 40 W).

18| Add 10 % (vol/vol) HCl until the solution is pH neutral.

19| Autoclave the resulting solution at 80 °C for 1 h.

20| Centrifuge the autoclaved solution at 1,6128*g* for 10 min at 25 °C, and designate the supernatant as GO-nS.
▲ **CRITICAL STEP** The resultant GO solution (Step 19) should be sterilized in order to be suitable for cell analysis, and the centrifugation steps should be carried out in a biology hood to ensure that the samples remain sterile.

21| Analyze the GO-nS samples using AFM, TEM (**Supplementary Fig. 1**), XRD (**Supplementary Fig. 2**), Raman spectroscopy (**Supplementary Fig. 3**) and FT-IR spectroscopy (**Supplementary Fig. 4**).
■ **PAUSE POINT** At this point, the solid can be stored at 4 °C for at least 3–4 weeks.

***In vitro* detection of multiple nucleotides with the aptamer/GO-nS complex**
22| Use option A for the single-aptamer/GO-nS complex, or use option B for the dual-aptamer/GO-nS complex. In addition, you could also perform an assay to demonstrate that the GO-nS complex protects the aptamers from endonuclease cleavage (**Box 1** and **Table 2**).
▲ **CRITICAL STEP** As far as possible, keep the plates in a dark place (usually we use foil to wrap the bottle or the 96-well plate, and we place bottles or plates in a cabinet or in drawers) to protect the fluorophores from photobleaching.
(A) Quenching ability test for single-aptamer and single-nucleotide detection based on the single-aptamer/GO-nS complex ● TIMING 100 min

(i) To a 96-well, flat-bottomed polypropylene black plate, add 85, 86, 87, 88, 88.5, 89, 89.5 and 90 μl of binding buffer; next, add 0, 0.5, 1, 1.5, 2, 3, 4 and 5 μl of a 100 μg/ml GO-nS solution and 10 μl of 100 nM ATP aptamer or GTP aptamer stock solution in binding buffer (see Reagent Setup) to a final concentration of 0, 0.5, 1, 1.5, 2, 3, 4 and 5 μg/ml GO-nS with a total volume of 100 μl in each well. Shake the plate gently for 5 min to ensure good mixing. This test should be performed in triplicate; there should be three data points for each GO-nS concentration measured.

Box 1 | Cleavage protection of aptamer probes by GO-nS (optional) ● TIMING 4.5 h

1. Clean and dry the electrophoresis devices.
2. Place the device on a horizontal table. Connect the apparatus with power supply correctly. Prepare the electrophoresis system for running a gel.
3. Prepare a 3.5% (wt/vol) agarose-TBE sol-gel for the electrophoresis experiments.
4. Allow 30 min for the gel to polymerize.
5. Prepare the DNA sample and DNase I reaction samples while the gel is polymerizing according to the guidelines in **Table 2**.
6. Pull out the comb gently and quickly. Next, add TBE buffer into the box until the buffer covers the gel well.
7. Load the DNA ladder and samples into the gel. To separate the aptamer and the GO-nS, electrophoresis is carried out right after heating of the samples at 90 °C for 5 min.
8. Perform electrophoresis in 10 mM TBE buffer at 100 V for 1 h.
9. Stain the gel by 5,000-fold diluted SYBR Green nucleic acid gel stain in 10 mM TBE for 30 min.
10. Rinse the gel with 10 mM TBE buffer three times to completely remove the stain molecules.
11. Turn on the NucleoVision imaging system and allow at least 20 min of warm-up time before use.
12. Start the GelExpert 2.0 software. Image the gel via NucleoVision imaging system using UV irradiation and capture the photos via the GelExpert 2.0 software (**Supplementary Fig. 7**).

? TROUBLESHOOTING

(ii) Acquire the fluorescence intensity of FAM or Cy5 (excitation/emission) via a Tecan Safire 2 microplate reader 5 min after the mixing (**Fig. 3a**,**b**).

(iii) By using a new plate, add 3 μl of 100 μg/μl GO-nS solution and 10 μl of 100 nM ATP aptamer or GTP aptamer stock solution in binding buffer to a final concentration of 100 nM single-aptamer/GO-nS complex. Add ATP or GTP stock solution (2 mM or 20 mM) to a final ATP or GTP concentration of 0, 0.01, 0.1, 0.5, 1, 1.5 and 2 mM, respectively (the total volume of the reaction solution is 100 μl in each well). Place the plate on a gentle shaker to ensure good mixing and then incubate the plate at 25 °C for 1 h.

(iv) Acquire the fluorescence intensity of FAM or Cy5 (excitation/emission) via a Tecan Safire 2 microplate reader. This test should be performed in triplicate.

(v) Analyze the data of ATP or GTP detection based on single-aptamer/GO-nS complex, as shown in **Supplementary Figure 5**. After incubation with ATP or GTP for 60 min, FAM or Cy5 fluorescence should be recovered linearly over the range of ATP or GTP concentrations.

? TROUBLESHOOTING

(B) Quenching ability test for dual-aptamer and multiple-nucleotide detection based on a multiple-aptamer/GO-nS complex ● TIMING 2 h

(i) To a 96-well, flat-bottomed polypropylene black plate, add 75, 76, 77, 78, 78.5, 79, 79.5 and 80 μl of binding buffer, 10 μl of 100 nM ATP aptamer–FAM and 10 μl of 100 nM GTP aptamer–Cy5 simultaneously in binding buffer; next, add 100 μg/ml GO-nS solution to a final concentration of 0, 0.5, 1, 1.5, 2, 3, 4 and 5 μg/ml GO-nS with a total binding buffer of 100 μl in a 96-well plate. Shake the plate gently for 5 min to ensure thorough mixing. Perform the test in triplicate by representing each solution in three wells of the plate.

TABLE 2 | Experimental recommendations for electrophoretic reagent setup.

Gel lane no.	1	2	3	4	5	6	7
10 bp DNA ladder	1	—	—	—	—	—	—
1 μM ATP aptamer (μl)	—	1	1	1	1	1	1
100 μg/μl GO-nS solution (μl)	—	—	—	—	1	1	1
4 U/μl DNase I (μl)	—	—	1	1	—	1	1
Cyan/orange loading buffer (μl)	3.3	3.3	3.3	3.3	3.3	3.3	3.3
Sterile water (μl)	15.7	15.7	14.7	14.7	14.7	13.7	13.7
Total volume (μl)	20	20	20	20	20	20	20
Reaction time (min)[a]	—	—	15	40	—	15	40

[a]Reaction temperature for DNase I is 37 °C.

(ii) Acquire the fluorescence intensity of FAM and Cy5 (**Fig. 3c**).

(iii) By using a new plate, add 10 µl of 100 nM ATP aptamer–FAM and 10 µl of 100 nM GTP aptamer–Cy5 simultaneously in binding buffer; next, add 5 µl of a 100 µg/ml GO-nS solution to a final concentration of 5 µg/ml GO-nS. Repeat the option eight times to prepare eight sample wells.

(iv) Next, add ATP, GTP, CTP or TTP stock solutions, respectively, to each well to make a final concentration of 0.5 mM and 2 mM for each nucleotide. Place the plate on a gentle shaker gently to ensure good mixing and then incubate it at 25 °C for 1 h.

(v) Acquire fluorescence intensity of FAM and Cy5 (excitation/emission) via a Tecan Safire 2 microplate reader.

(vi) Analyze the data obtained from the fluorescence spectrum, an example of which shown in **Figure 4**.

? TROUBLESHOOTING

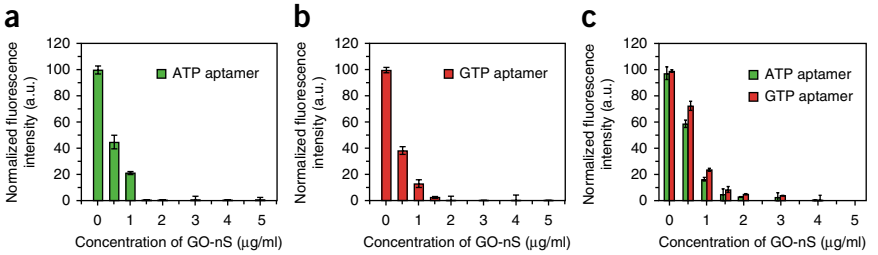

Figure 3 | Fluorescence quenching ability test of GO-nS for single or multiple aptamer probes. (**a**–**c**) Normalized fluorescence intensity of 100 nM ATP aptamer–FAM (**a**), 100 nM GTP aptamer–Cy5 (**b**) and the mixture of 100 nM ATP aptamer–FAM and 100 nM GTP aptamer–Cy5 (**c**) versus the concentration of GO-nS from 0.5 to 5 µg/ml in reaction buffer. Error bars show mean ± s.d. and were obtained from three parallel experiments. Excitation wavelength for FAM: 470 nm; for Cy5: 650 nm. a.u., arbitrary units. This figure is adapted with permission from our previously published work, copyright 2013 American Chemical Society[38].

***In situ* live-cell imaging of multiple nucleotides** ● **TIMING 1.5–2.5 d**

23| For live-cell imaging experiments, seed 6 × 10^4 cells in 500 µl of complete MEM medium into each glass-bottomed microscope dish, and incubate the dishes at 37 °C for 24–36 h to ensure that each well has reached 50–70% confluency before adding the GO-nS or aptamer/GO-nS complex (Step 24). Each dish contains 1 ml of culture medium. Keep the cell dish as a control, without any options, until confocal imaging.

24| Prepare 5 ml of cell culture medium (see Reagent Setup) with a GO-nS or aptamer/GO-nS complex to obtain a final concentration of 100 nM. For GO-nS incubated with cells, dilute the stock solution of GO-nS (Step 20) with cell culture medium to obtain a final concentration of 3 µg/ml. For the ATP aptamer/GO-nS complex, prepare a 100-µl solution of 100 nM ATP aptamer–FAM with 3 µg/ml GO-nS; for the GTP aptamer/GO-nS complex, prepare a 100-µl solution of 100 nM GTP aptamer–Cy5 with 3 µg/ml GO-nS; for a multiple aptamer/GO-nS complex, prepare a 100-µl solution of 100 nM ATP aptamer–FAM, 100 nM GTP aptamer–Cy5 and 100 nM random DNA–Alex546N with 5 µg/ml GO-nS. Because of the adsorption interaction between aptamers or single-stranded DNA and GO-nS, an aptamer/GO-nS nanocomplex can be formed through self-assembly while completely mixing (optional). At this point, you can also prepare samples to perform the cell viability assay (**Box 2**) to check that none of the components are toxic to cells.

25| When the cells have reached ~50–70% confluency, remove the culture medium from each dish and wash the cells with 1 ml of PBS once.

▲ **CRITICAL STEP** The washing PBS buffer should be prewarmed to 37 °C.

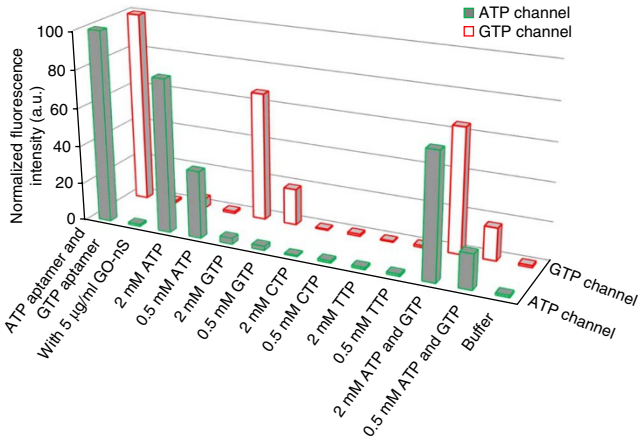

Figure 4 | Selective response to ATP, GTP, CTP and TTP based on the aptamer/GO-nS sensing platform by recording the respective fluorescence channel. Green with gray fill indicates the FAM channel; red indicates the Cy5 channel. Normalized fluorescence intensity of the mixture of 100 nM ATP aptamer–FAM and 100 nM GTP aptamer–Cy5 in reaction buffer is shown in (ATP aptamer and GTP aptamer). After injection of GO-nS, fluorescence was quenched (with 5 µg/ml GO-nS). By incubating ATP aptamer–FAM/GTP aptamer–Cy5/GO-nS complexes with 0.5 and/or 2 mM ATP, GTP, CTP or TTP for 1 h at 25 °C, respectively (shown in the corresponding columns), fluorescence recovery was obtained. Excitation wavelength for FAM: 470 nm; for Cy5: 650 nm. This figure is adapted with permission from our previously published work, copyright 2013 American Chemical Society[38].

Box 2 | Determine the cell viability by performing trypan blue exclusion (optional)

● TIMING 3–6 d

1. Culture the cells in a cell dish for 12–24 h in 5% CO_2 at 37 °C and grow the cells to confluence for 24–48 h before subculture.
2. Incubate the cells with 1 to 9 μg/ml GO-nS for 12, 24 and 72 h, respectively, after seeding for 24 h in 5% CO_2 at 37 °C
3. Trypsinize the cells and seed them at a density of 5 × 10^4 cells per well into a 12-well flat-bottomed plate.
4. Determine the cell viability after labeling by performing trypan blue exclusion according to the manufacturer's protocol (0.05% (wt/vol) trypan blue staining for 5 min).

? TROUBLESHOOTING

26| Add different cell culture media into different cell dishes according to the table given below.

Control test	Use the above-mentioned cell culture methods to grow the cells
Coculture with aptamer probe	Incubate the cells with 1 ml of complete MEM cell medium consisting of 100 nM ATP aptamer-FAM, 100 nM GTP aptamer–Cy5 and 100 nM random DNA–Alex546N for 6 h at 37 °C under 5% CO_2
Coculture with multiple aptamer/GO-nS complexes	Mix ATP aptamer–FAM/GO-nS, GTP aptamer–Cy5/GO-nS and random DNA–Alex546N/GO-nS nanocomplex solutions to a final concentration of 100 nM with 5 μg/ml GO-nS. Incubate the cells with 1 ml of complete MEM cell medium consisting of 100 nM ATP aptamer–FAM/GO-nS, 100 nM GTP aptamer–Cy5/GO-nS and 100 nM random DNA–Alex546N/GO-nS for 6 h at 37 °C under 5% CO_2

27| Incubate the cells at 37 °C, 5% CO_2 for 6 h. Remove the culture medium from each dish and rinse the cells three times with fresh PBS buffer.

28| Turn on the Zeiss LSM 710 NLO laser-scanning confocal microscope and allow at least 60 min of warm-up time before use. Start the ZEN 2009 light program.

29| Obtain fluorescence images using an equal exposure time for all experiments with the appropriate filter. Expected imaging results can be found in **Figure 5**. The maximum excitation wavelength and maximum emission wavelengths for ATP aptamer–FAM are 495 and 520 nm (green), for GTP aptamer–Cy5 they are 650 and 670 nm (red) and for random DNA–Alex546N they are 556 and 573 nm (orange).

30| To perform an experiment in which cells are incubated with different concentrations of aptamer/GO-nS nanocomplex, prepare 5 ml of cell culture medium with the multiple aptamer/GO-nS complex to make a final concentration of 25, 50, 100 and 200 nM, respectively. Next, add different cell culture media into different cell dishes and incubate them at 37 °C, 5% CO_2 for 6 h. Remove the culture medium from each dish and rinse the cells three times with fresh PBS buffer solution. Obtain fluorescence images using equal exposure time.

▲ CRITICAL STEP All of the imaging parameters should be kept the same in order to compare the signal in each image.

? TROUBLESHOOTING

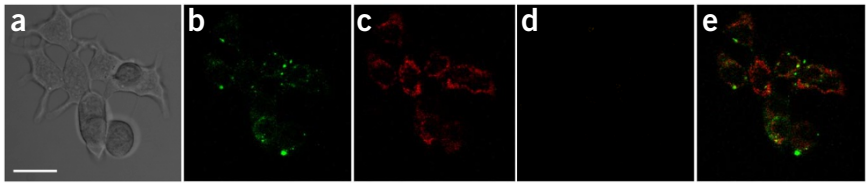

Figure 5 | Confocal images of *in situ* visualization for ATP and GTP. Images represent MCF-7 cells incubated in completed EME medium for 6 h with 100 nM ATP aptamer–FAM/GO-nS, 100 nM GTP aptamer–Cy5/GO-nS and 100 nM random DNA-Alex546N/GO-nS. (**a**–**d**) Panel **a** is the bright-field image of MCF-7 cells, panel **b** is the channel of FAM, panel **c** is the channel of Cy5 and panel **d** is the channel of Alex546N. The merged fluorescent panels for confocal images are shown in panel **e**. Images were captured by confocal microscopy after extensive washing of cells with PBS. Scale bar, 10 μm.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reason	Solution
16	The color of the solution becomes dark brown or an agglomeration forms	The ice in the ice bath has melted or the autoclave temperature is too high	Prepare sufficient ice before the sonicating step; set the autoclave temperature at 75–80 °C
22A(v), B(viii)	Unable to obtain the expected fluorescence spectrum of aptamer-dye probes quenched with GO-nS	Buffer solution is expired The solutions are not mixed well	Prepare new binding buffer to repeat the experiments If centrifuge tubes are used, mix the aptamer-dye with GO-nS in the binding buffer completely by using a vortex; if you are using a 96-well plate, mix the solution in each well completely with a pipettor
30	Unable to obtain the confocal image of cellular ATP, GTP or ATP and GTP simultaneously	Cells are not in good condition, or the aptamer/GO-nS nanocomplexes are not prepared well	Prepare aptamer/GO-nS nanocomplexes carefully before sub-culture, and mix the solution completely just before adding Culture healthy cells again and repeat these steps
Box 1	Too many bubbles	Agarose-TBE solution is too hot or it was not boiled for long enough	Go to Step 32 to prepare a new agarose-TBE solution, boil it for twice as long, and then cool it down to ~60 °C
Box 2	Cell viability is poor	GO-nS quality is poor Sterilization of GO-nS has failed	Go to Step 21 to do the quality control test, to make sure the GO-nS is up to the standard Repeat the sterilization to try the cell viability assay; if it fails again, please go back to Step 1 to make new GO and GO-nS samples

● TIMING

Steps 1–14, synthesis of graphene oxide: 7–14 d
Steps 15–20, preparation of GO-nS: 7 h
Step 21, characterization of GO-nS: 8 h
Step 22A, GO-nS quenching ability test: 100 min
Step 22B, nucleotide detection and selectivity test: 2 h
Steps 23–30, *in situ* live-cell imaging of multiple nucleotides, pre–cell culture (1–2 d), co–cell culture (6–8 h) and confocal imaging (1–2 h)
Box 1, cleavage protection of aptamer probes by GO-nS (optional), 4.5 h
Box 2, determine the cell viability by performing trypan blue exclusion (optional), 3–6 d

ANTICIPATED RESULTS

Preparation and characterization of the aptamer/GO-nS nanocomplex

Before using the aptamer/GO-nS nanocomplex for simultaneous bioimaging of cellular ATP and GTP, the aptamer/GO-nS nanocomplex should be characterized. Generally, GO-nS for biological applications should have a small and homogeneous size and good water solubility. GO-nS should also possess efficient fluorescence quenching performance to satisfy our sensing platform. A representative AFM and TEM images (**Supplementary Fig. 1**), an XRD (**Supplementary Fig. 2**), a Raman spectrum (**Supplementary Fig. 3**), and an FT-IR spectrum (**Supplementary Fig. 4**) are shown in the supplementary information.

ATP aptamer–FAM and/or GTP aptamer–Cy5 quenched with GO-nS are shown in **Figure 3**. As can be seen, a higher GO-nS concentration up to 5 μg/ml is ideal for quenching ATP aptamer–FAM and GTP aptamer–Cy5 concurrently (**Fig. 3c**). After incubation of 10 μM to 2.5 mM ATP or GTP, fluorescence recovery of up to 85.7% should be obtained owing to the specificity between aptamers and their targets. The loop-structured assembly of the aptamer and target no longer has good affinity for GO-nS; therefore, the fluorophores are further away from the quencher (GO-nS) surface and the fluorescence of the dyes can be detected.

We carried out a test of aptamer versus nucleotide specificity by specifically monitoring the appropriate excitation/emission wavelengths for FAM and Cy5 dyes (FAM excitation 495 nm/emission 520 nm; Cy5 excitation 650 nm/emission 670 nm). We observed selective release of ATP aptamer or GTP aptamer owing to the high specific interaction between the aptamer and the target. However, for CTP and TTP, no obvious change was obtained (**Fig. 4**). From **Figure 4**, we can confirm

that multiple aptamers could be adsorbed onto GO-nS while retaining good specificity for their respective triphosphates to dissociate from the aptamer/GO-nS complex.

Incubation of cultured cells with multiaptamer/GO-nS complexes

By incubating MCF-7 cells with multiaptamer/GO-nS complexes consisting of ATP aptamer–FAM, GTP aptamer–Cy5 and random DNA–Alex546N for 6 h, typical confocal images of living MCF-7 cells can be obtained by following the described protocol for cellular ATP and GTP imaging, and examples are shown in **Figure 5**. **Figure 5a** shows the bright-field image of MCF-7 cells treated with the aptamer/GO-nS nanocomplex, whereas **Figure 5b**,**c** shows the fluorescence signal derived from ATP aptamer–FAM and GTP aptamer–Cy5. **Figure 5d** shows little or no fluorescence signal from the random DNA aptamer (random DNA–Alex546N was designed as a reference probe to evaluate the specificity of this platform in living cells). These images can be combined into a single image via the different color channels shown in **Figure 5e**. In addition, it is very hard to observe fluorescence signals in the cells incubated without GO-nS. From **Figure 5**, we can confirm that the GO-nS–sensing platform can deliver multiple aptamer probes into living cells and successfully realize the *in situ* visualization of ATP and GTP simultaneously. Moreover, application of aptamer-FAM/GO-nS in real-time monitoring demonstrates that the aptamer/GO-nS is evident in the intracellular region (**Supplementary Fig. 6**). On the basis of our design, as culture time elapses, more aptamers will enter into the cells and then form duplexes with cellular ATP, followed by fluorescence recovery. Real-time monitoring of cellular ATP in JB6 cells (mouse epithelial cells, JB6 Cl 41-5a) has been performed by using a wide-field microscope in our previous work[36]. When culture time took up to 4 h, the fluorescence intensity of JB6 cells incubated with aptamer-FAM/GO-nS increased notably compared to the cells cultured with GO-nS or aptamers only. In general, fluorescence intensity inside cells derived from aptamer-FAM/GO-nS increases as culture time elapses. Hence, real-time or even online observation of the intracellular transportation, as well as cellular ATP or GTP monitoring, can be performed for up to 8 h after incubation with the present protocol. The optimized time window for cell imaging is 8–8.5 h after incubation.

Stability to treatment with DNase

Further investigation of GO-nS for biosensing applications has been demonstrated using electrophoresis. DNase I, which can nonspecifically cleave single- and double-stranded DNA, is used to simulate enzymatic cleavage functions in living cells. As shown in **Supplementary Figure 7**, incubation of ATP aptamer–FAM with DNase I (0.2 units/μl) for 15 or 40 min shows a vivid migration due to the enzymatic cleavage (lanes 3 and 4), relative to ATP aptamer–FAM control without any DNase I (lane 2). In contrast, the ATP aptamer–FAM/GO-nS complex is difficult to cleave with DNase I after 15 or 40 min of incubation, as shown in lanes 6 and 7 (the aptamer-FAM/GO-nS without DNase is shown in lane 5). We use the 10-bp DNA ladder as marker and load it in lane 1. The results demonstrate that single-stranded DNA is effectively and promptly adsorbed onto GO-nS, and that the DNA is strongly restrained on a graphene oxide surface, which prevents the DNase from approaching the DNA molecule.

Cell viability assays

Meanwhile, for *in situ* target monitoring in living cells, the aptamer/GO-nS complex is expected to be of good biocompatibility and low toxicity. Consequently, we investigated whether GO-nS reduced cell viability in MCF-7 cells as an initial test case (**Supplementary Fig. 8**). GO-nS showed negligible effects on cell viability at concentrations ≤7 μg/ml. We observed toxicity at the highest concentration (9 μg/ml) by 72 h. The results demonstrate that GO-nS exhibited negligible effects on the growth of MCF-7 cells with concentrations lower than 7 μg/ml when the postculture time was <72 h.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS Y.W., Z.L., J.L. and Y.L. designed the protocol and carried out the experiments. Y.W., L.T. and J.L. wrote the manuscript.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

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