

Electronic Supplementary Information (ESI) for:

Circular DNA: A Stable Probe for High Efficient mRNA Imaging and Gene Therapy in Living Cells

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Experimental Section

Chemicals. All oligonucleotides (shown in Table S1) were synthesized and HPLC purified by Sangon Biotechnology Co., Ltd (Shanghai, China). Graphene oxide water dispersion was purchased from XFNANO Materials Tech Co., Ltd (Nanjing, China). LysoTracker Green and Hoechst 33342 were purchased from Thermo Fisher Scientific Inc (USA). T4 DNA ligase was obtained from New England Biolabs Co., Ltd (Beijing, China) and Exonuclease I and III were purchased from TaKaRa Biotechnology Co., Ltd (Dalian, China). Ultrapure water obtained from a Milli-Q water purification system (18.2 MΩ resistivity, Millipore). All reagents were received and used without further purification.

Table S1 The detailed information of oligonucleotides

Name	Detailed sequence information
survivin probe-Cy3 ^a	5'- CCTGAT <u>CCCAGCCTTCCAGCTCCTTGT</u> (Cy3) <u>TCCCAGCCTTCCA</u> <u>GCTCCTTGTTCCCAGCCTTCCAGCTCCTTGTTAGTCGGTA</u> -3'
survivin probe-Cy5	5'- CCTGAT <u>CCCAGCCTTCCAGCTCCTTGT</u> (Cy5) <u>TCCCAGCCTTCCA</u> <u>GCTCCTTGTTCCCAGCCTTCCAGCTCCTTGTTAGTCGGTA</u> -3'
survivin target	5'-CAAGGAGCTGGAAGGCTGGG-3'
survivin single base mismatched target	5'-CAAGGAGCTG C AAGGCTGGG-3'
<i>c-raf</i> probe-Cy5 ^b	5'- CCTGAT <u>TCCCGCCTGTGACATGCATTT</u> (Cy5) <u>TTCCCGCCTGTGA</u> <u>CATGCATTTTTCCCGCCTGTGACATGCATTTT</u> AGTCGGTA-3'
<i>c-raf</i> target	5'-AATGCATGTCACAGGCGGGA-3'
<i>c-raf</i> probe with single base mutation-Cy3	5'- CCTGAT <u>TCCCGCCTATGACATGCATTT</u> (Cy3) <u>TTCCCGCCTATGA</u> <u>CATGCATTTTTCCCGCCTATGACATGCATTTT</u> AGTCGGTA-3'
<i>c-raf</i> probe with three base mutation-Cy3	5'- CCTGAT <u>TCCCACCTATGACATACATTT</u> (Cy3) <u>TTCCCACCTATGA</u>

	CAT <u>A</u> CATTTTTTCC <u>C</u> ACCTATGACAT <u>A</u> CATTTTAGTCGGTA-3'
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Underlined letters of probe denote the complementary sequence with target. Red letters represent mutant bases in mismatched target sequences.

^a The target site of survivin probe sequence is NO.304-NO.323 on survivin mRNA, which is the 2655-bp mRNA (GenBank accession number NM001168).

^b The target site of *c-raf* probe sequence is NO.2771-NO.2790 on *c-raf* mRNA, which is the 3282-bp mRNA (GenBank accession number NM002880).

Apparatus. The UV-vis absorption spectra were recorded using a SH-1000 Lab microplate reader (Corona Electric, Ibaraki, Japan). The fluorescence spectra and kinetics were measured on an Agilent Cary Eclipse Fluorescence Spectrophotometer (USA). The fluorescence images of cells were taken on a Nikon A1 confocal fluorescence microscope (Ja-pan). Gel electrophoresis was imaged by Bio-Rad Chemi-Doc™ Touch Imaging System (USA).

Cell lines and Cell Culture. HeLa cells (human cervical epithelial carcinoma) and MCF-10A (normal human epithelial mammary) cells were purchased from ATCC (Manassas, VA). HeLa cells were cultured in RPMI 1640 medium (HyClone) supplemented with 10% fetal bovine serum (FBS, Gibco) and 100 IU/mL penicillin-streptomycin at 37 °C in 5% CO₂ atmosphere. MCF-10A cells were grown in MEGM kit (Lonza, Switzerland) and cholera toxin (Sigma Aldrich, USA) at 37 °C in 5% CO₂ atmosphere.

Preparation of cDNA. Circularization was carried out by self-ligation of ssDNA. The self-ligation reaction was conducted at 16 °C overnight catalyzed by T4 DNA ligase in the presence of proper primer sequence, and then the ligase was denatured at 65 °C for 10 min. After that, unreacted ssDNA and primers were degraded by exonuclease I and exonuclease III at 37 °C for 1 h followed by denatured at 90 °C for 10 min. Finally, the cDNA was purified with QIAEX II Gel Extraction Kit (Qiagen, Germany) and the concentration of cDNA was determined with UV-vis spectrophotometry prior to usage.

Stability analysis of DNA in serum. 2 μM cDNA or ssDNA were incubated with RPMI 1640 with 10% FBS at 37 °C for different times. At each time points, samples were heated for 5 min at 95 °C to denature the enzyme in FBS and stored at -80 °C for further experiment.

Polyacrylamide gel electrophoresis (PAGE) assay. Urea-denatured PAGE and non-denatured PAGE were performed to evaluate different characteristics of cDNA. The former was used to identify the

synthesis of cDNA. The latter was used to verify the hybrid between cDNA and target mRNA and evaluate the stability of DNA in serum. 10 μ L samples were mixed with 2 μ L 6 \times loading buffer and loaded into gel in 1 \times TBE buffer at 4 $^{\circ}$ C. Finally, gels were stained by GelRed and analyzed with imaging system under the UV-trans model after exposure for 2.0 s.

Synthesis of DNA/GO. cDNA or ssDNA were diluted into 100 nM with binding buffer (PBS containing 10 mM MgCl₂), GO solution was added and incubated at room temperature for 10 min.

***In vitro* fluorescence experiments.** In these experiments, Cy5-labeled cDNA or ssDNA was used for fluorescence spectra measurement, while the excitation and emission wavelength were 640 nm and 650-750 nm, respectively. For GO dosage optimization, GO with different concentration ranged from 1 μ g/mL to 4 μ g/mL were added into 100 nM cDNA in binding buffer. After incubation at 37 $^{\circ}$ C for 10 min, the fluorescent intensity at 665 nm were measured and recorded as a curve. For LOD measurement, cDNA/GO or ssDNA/GO was diluted to the concentration of 100 nM in binding buffer and co-incubated with different concentration of the targets ranged from 1 nM to 1000 nM. After incubation for 30 min at 37 $^{\circ}$ C, the fluorescence emission spectra were recorded in 1 nm increment. For specific detection, kinetics measurements with different targets (completely complementary and single-base mismatch) of 300 nM were employed for 2 h. The investigation was performed at room temperature. For target detection in complex matrix, cDNA/GO or ssDNA/GO was dispersed in solution containing 10% serum with or without 300 nM targets. After incubation at 37 $^{\circ}$ C for 30 min, the emission spectra were recorded. For the FP assay, 100 nM cDNA or ssDNA was incubated at 37 $^{\circ}$ C for 30 min in the presence and absence of GO, followed by the addition of 100 nM target. The level of FP was recorded using fluorescence spectrometer. The average and standard deviation were obtained by 3 parallel experiments in each trial.

Cellular confocal fluorescence microscopy. 2.5 \times 10³ cells (HeLa or MCF-10A) were seeded in a 35 mm confocal dish (NEST Biotechnology Co. Ltd, China) and grown for 24 h before experiments. After washed twice with PBS, 300 nM dye-labeled cDNA/GO or ssDNA/GO was added. After incubation at 37 $^{\circ}$ C for different times, the cells were stained by LysoTracker Green (50 nM) and Hoechst 33324 (1:1000 dilution) according to the manufacture's protocol. Finally, the cells were washed twice and sent for fluorescent confocal imaging.

Cytotoxicity assay. The effect of cDNA/GO or ssDNA/GO on gene therapy was evaluated using the Cell

Counting Kit-8 (Dojindo, Japan). Briefly, HeLa or MCF-10A cells were planted in 96-well plate at a density of about 8×10^3 cells per well and incubated for 24 h, then cDNA/GO or ssDNA/GO with different concentration were added to designated wells and incubated for another 24 h. After replacing the cell culture with fresh cell medium, 10 μ L CCK-8 solution was added to each well for additional 1 h incubation. Finally, the absorbance of each well at 450 nm was measured by a microplate reader to determine the cell viabilities.

Western blot assay. HeLa cells (10^5 cells/well) were plated in 12-well plates for 24 h, 300 nM cDNA/GO or ssDNA/GO was added for gene regulation. 24 h after interference, cells were harvested and total protein extracts were prepared using Cell Lysis Buffer for Western (Beyotime Biotech) and Protease Inhibitor Cocktail Set III (Merk). Then, the concentrations of protein were quantified with BCA Protein Assay Kit (Beyotime Biotech.) following the manufacturer's instruction. Then, 15 μ g protein extracts for each sample was mixed with loading dye and electrophoresed on a 10% SDS polyacrylamide gel and transferred to polyvinylidene difluoride (PVDF) membranes (BioRad). Immunoblotting was carried out with an anti-survivin monoclonal antibody or anti-raf1 antibody (Abcam) and using rabbit anti-mouse IgG/HRP (Cell Signaling Technology) as a secondary antibody. Finally, peroxidase activity was detected by using the enhanced chemiluminescent substrate HRP detection reagent (Thermo Scientific).

Supporting information of calculation

The binding kinetics was measured to estimate the affinity difference between cDNA and ssDNA in combination with GO. As shown in Fig. S2a, at the low GO concentration range (0-0.8 $\mu\text{g}\cdot\text{mL}^{-1}$), K_A was calculated from the intercept of the plots. The binding constant (K_A) was calculated according to the following equation¹

$$\lg\left(\frac{F_0}{F} - 1\right) = \lg K_A + n \lg C_{GO}$$

In this equation, F_0 and F are emission intensities of Cy5-cDNA in the absence and presence of GO, respectively.

To illustrate the selectivity of cDNA/GO and ssDNA/GO against target recognition quantitatively, a selectivity coefficient α was introduced, which was defined as $\alpha = \frac{(S/B)_{ij}}{(S/B)_{i'j'}}$. Where $(S/B)_{ij}$ or $(S/B)_{i'j'}$ is the S/B value of the probe i in the presence of target j or j' .² The selectivity for the perfectly matched target was used as the standard ($\alpha = 1$). With regard to cDNA/GO and ssDNA/GO, selectivity coefficient of 0.313 and 0.745 was obtained respectively.

Reference:

1. S. Y. Bi, L. Ding, Y. Tian, D. Q. Song, X. Zhou, X. Liu and H. Q. Zhang, *J. Mol. Struct.*, 2004, **703**, 37.
2. R. H. Yang, J. Y. Jin, Y. Chen, N. Shao, H. Z. Kang, Z. Xiao, Z. W. Tang, Y. R. Wu, Z. Zhu and W. H. Tan, *J. Am. Chem. Soc.*, 2008, **130**, 8351.

Supporting Figures

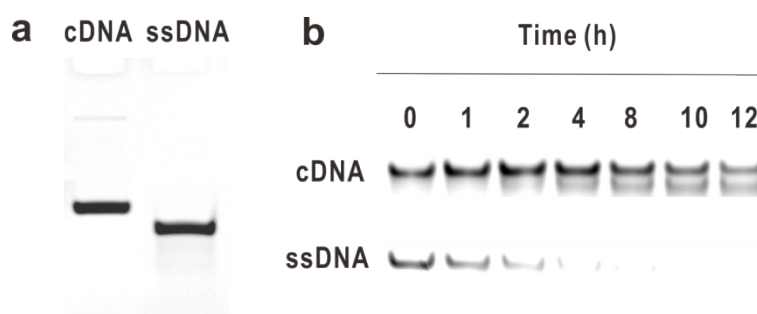


Fig. S1 (a) Urea-denatured PAGE image of as-prepared cDNA and ssDNA. (b) Stability analysis of cDNA and ssDNA in cellular cultural media containing 10% FBS for different times, as determined by non-denatured PAGE.

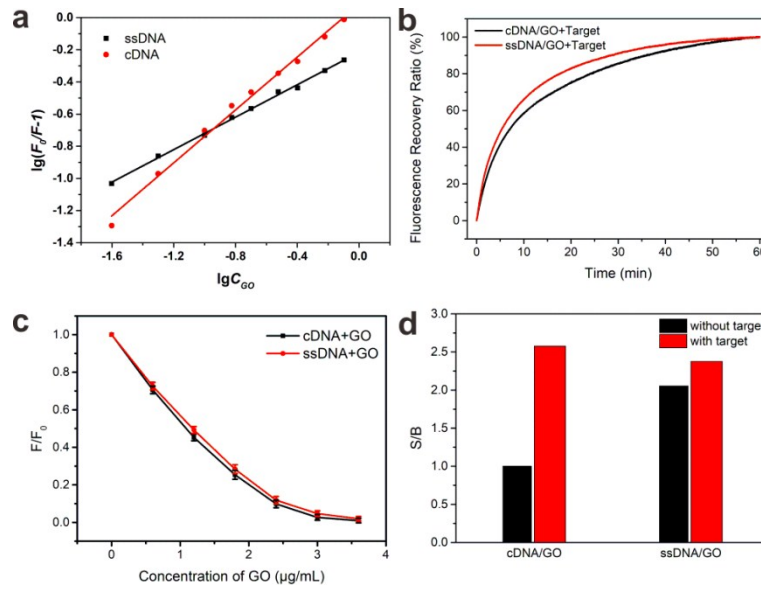


Fig. S2 (a) Plots of $\lg(F_0/F-1)$ vs. $\lg C_{GO}$ for the binding of cDNA and ssDNA with GO. (b) Time dependent fluorescence responses to target of cDNA/GO (black curve) and ssDNA/GO (red curve). (c) Fluorescent quenching ratio of Cy5-cDNA and Cy5-ssDNA by GO with different concentration. (d) Signal to background (S/B) of Cy5-cDNA/GO and Cy5-ssDNA/GO with (red column) or without (black column) target in complex matrix sample containing 10% FBS.

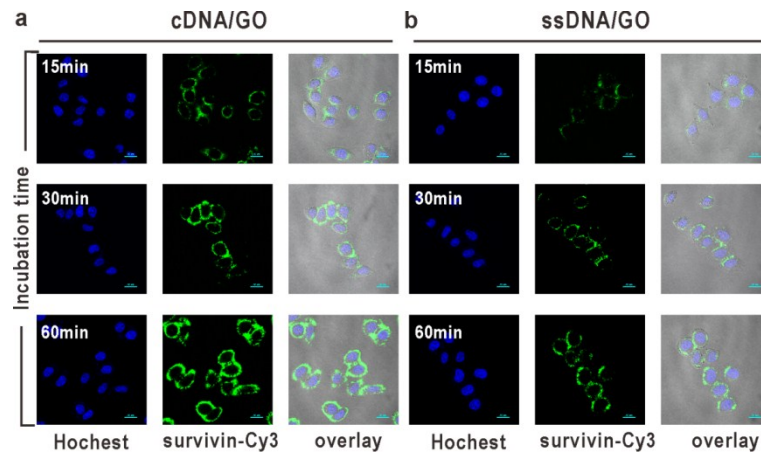


Fig. S3 CLSM images acquired of HeLa cells treated with survivin mRNA target (a) Cy3-cDNA/GO or (b) Cy3-ssDNA/GO for different time at 37 °C. Blue channels: cellular nucleus stained with Hoechst 33342; Overlay panels: merge of fluorescence and bright field image. Scale bar: 25 μm .

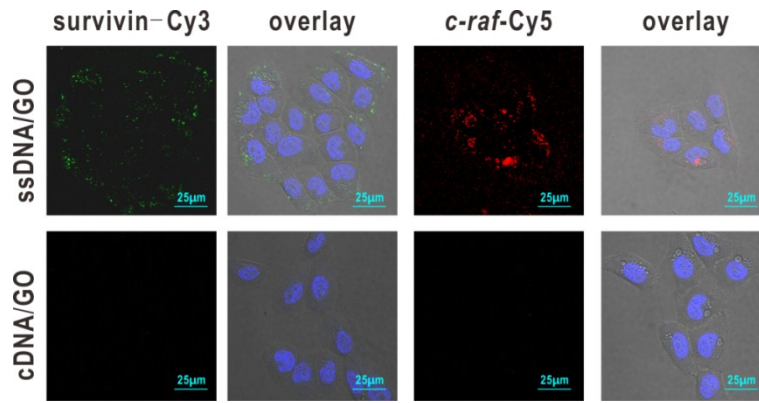


Fig. S4 CLSM images acquired of MCF-10A cells were treated with survivin and *c-raf* targeted cDNA/GO or ssDNA/GO for 60 min at 37 °C. Survivin targeted DNA was labelled by Cy3 and *c-raf* targeted DNA was labelled by Cy5. Overlay panels: merge of fluorescence and bright field image. Scale bar: 25 μm.

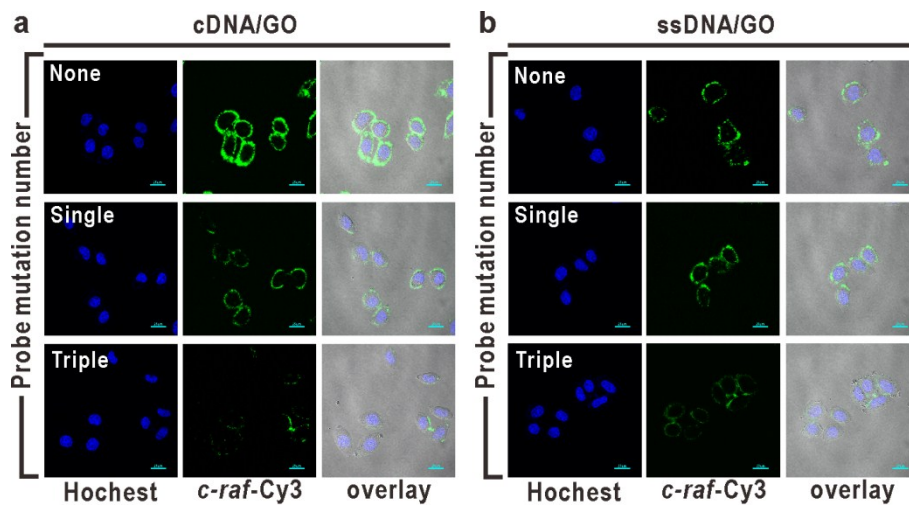


Fig. S5 CLSM images of HeLa cells treated with *c-raf* targeted (a) cDNA/GO or (b) ssDNA/GO with single base or triple base mutation for 60 min at 37 °C. Scale bars: 25 μm.

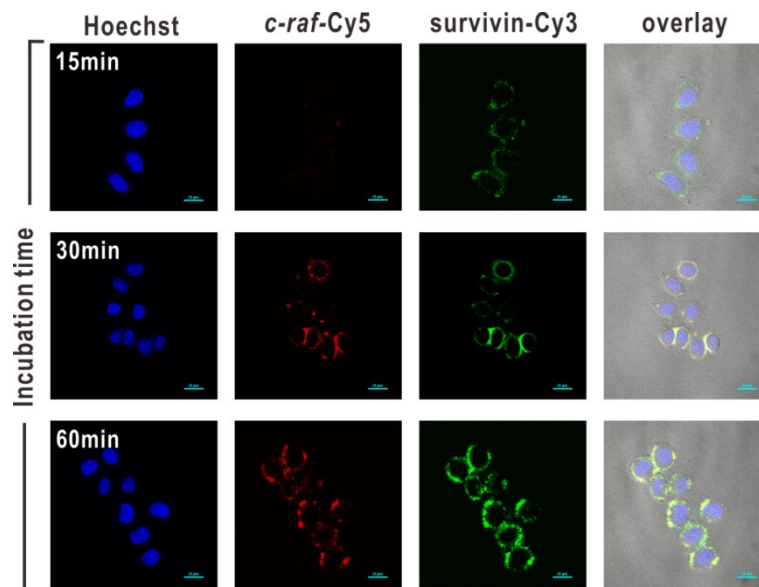


Fig. S6 CLSM images of HeLa cells treated with both survivin and *c-raf* targeted ssDNA/GO

for duplexed intracellular mRNA imaging. Blue channels are Hoechst 33324 fluorescence, red channels are Cy5-ssDNA targeted to *c-raf* mRNA, green channels are Cy3-ssDNA targeted to survivin mRNA and merged channels are overlay of fluorescence and bright field images. Scale bars: 25 μ m.

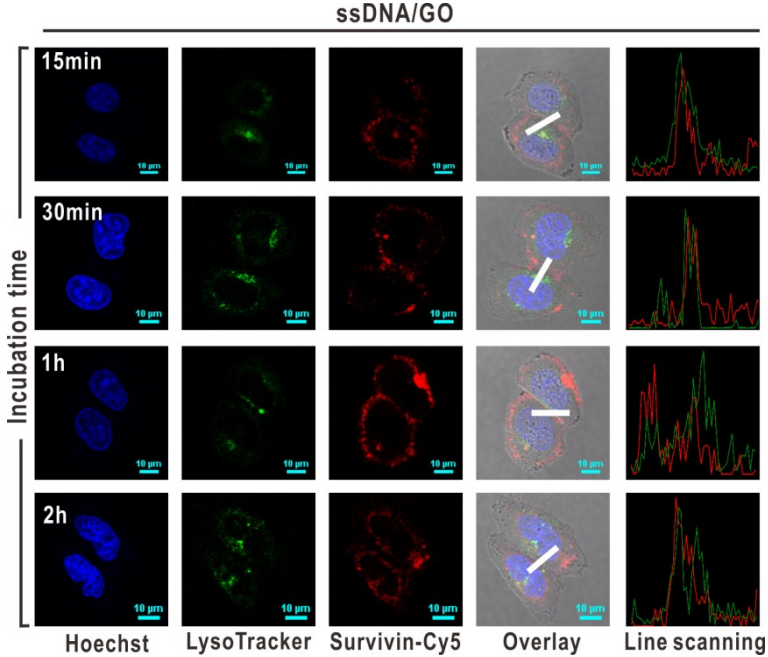


Fig. S7 Subcellular distribution of ssDNA/GO in HeLa cells. Green channels: endo/lysosomes were stained with LysoTracker; Blue channels: nucleic stained by Hoechst 33324; Red channels: survivin targeted Cy5-DNA; Overlay channels: merged image of fluorescence and bright field; Line scanning channels: fluorescence intensity profiles indicated the degree of overlap between DNA and endo/lysosomes, corresponding to the area in overlay channels that are highlighted by short white lines. Scale bar, 10 μ m.

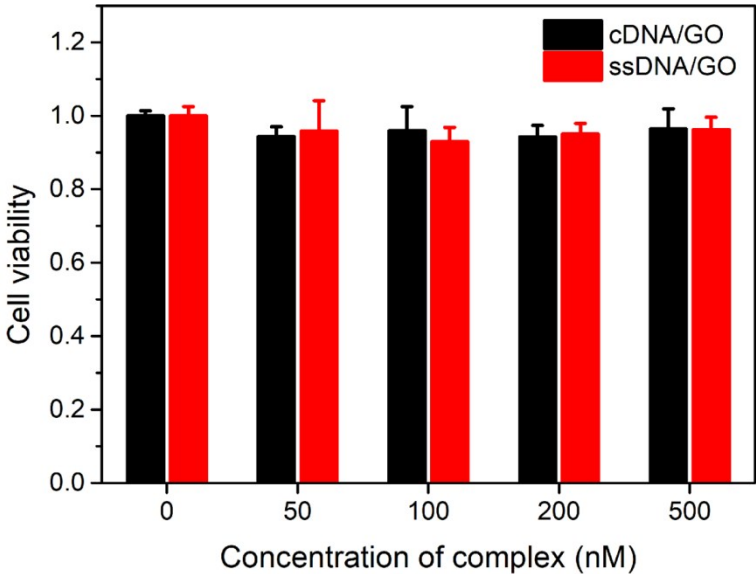


Fig. S8 MCF-10A cells proliferation inhibition induced by cDNA/GO or ssDNA/GO treatment at different concentrations for 24 h.

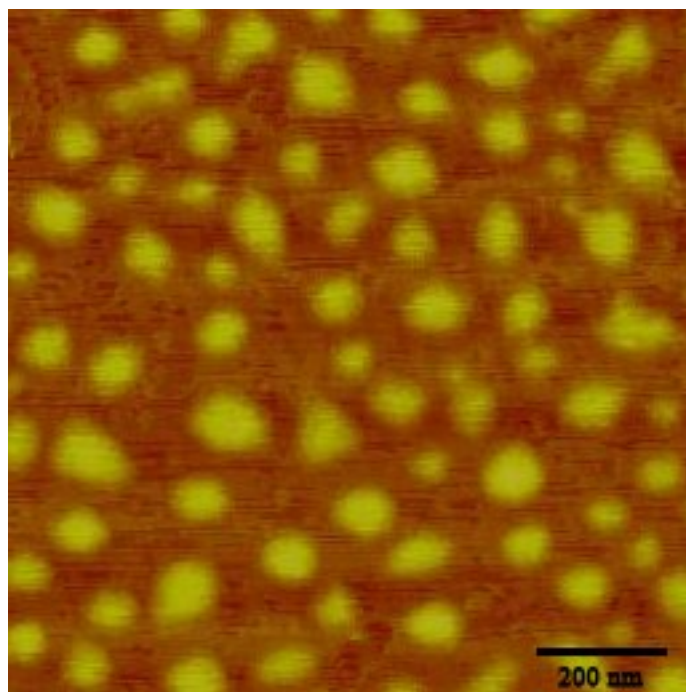


Fig. S9 AFM image of GO.