NPN uptake assay

Materials

N-Phenyl-1-naphthylamine (NPN), magnesium acetate tetrahydrate were obtained from MACKLIN® (Shanghai, China).
EDTA Na_2 was obtained from Solarbio® (Beijing, China).
Phosphate buffered saline (PBS) was obtained from TransGen Biotech (Beijing, China).
1.5M Tris-HCl was obtained from Beyotime® (Shanghai, China).
Anhydrous Ethanol was obtained from Sinopharm® (Beijing, China).
Hemin was obtained from MedChemExpress® (Shanghai, China).
E. coli ATCC25922 was obtained from hopebio® (Qingdao, China).
Materials needed for DNA origami (see preparation)

Procedures

1. Loading helper strands
   a. Prepare two microcentrifuge tubes on ice, labeling them as group A and group B. For group A, add M13, S-G4-x, S-x, and S-PAM-cap-x in the indicated amounts. For group B, add M13, S-G4-x, S-x, S-PAM-cap-x, and Apt-cap-x in the indicated amounts. The molar ratio of M13 (5nM) and helper strands (50 nM) is 1:10.

Group A: DNA origami G4 (DO G) (with S-PAM-cap-x)

Reagents	Volume
TAE/Mg^{2+}/K^{+} buffer	47μl
S-G4-x	0.25ul × 136 = 34μl
S-x	0.25ul × 50 = 12.5μl
S-PAM-cap-x	0.25ul × 6 = 1.5μl
M13	5μl

S-G4-15	**S-G4-66**	**S-G4-134**	**S-G4-182**	**S-133**
S-G4-16	**S-G4-67**	**S-G4-135**	**S-G4-183**	**S-156**
S-G4-17	**S-G4-68**	**S-G4-136**	**S-G4-184**	**S-157**
S-G4-20	**S-G4-69**	**S-G4-137**	**S-G4-185**	**S-180**
S-G4-21	**S-G4-70**	**S-G4-138**	**S-G4-186**	**S-181**
S-G4-22	**S-G4-71**	**S-G4-139**	**S-G4-187**	**S-194**
S-G4-26	**S-G4-74**	**S-G4-140**	**S-G4-188**	**S-198**
S-G4-27	**S-G4-75**	**S-G4-141**	**S-G4-189**	**S-199**
S-G4-28	**S-G4-76**	**S-G4-142**	**S-G4-190**	**S-203**
S-G4-29	**S-G4-77**	**S-G4-143**	**S-G4-191**	**S-204**
S-G4-30	**S-G4-78**	**S-G4-146**	**S-G4-195**	**S-24**

S-G4-31	**S-G4-79**	**S-G4-147**	**S-G4-196**	**S-25**
S-G4-32	**S-G4-80**	**S-G4-148**	**S-G4-197**	**S-48**
S-G4-33	**S-G4-81**	**S-G4-149**	**S-G4-200**	**S-49**
S-G4-34	**S-G4-82**	**S-G4-150**	**S-G4-201**	**S-72**
S-G4-35	**S-G4-83**	**S-G4-151**	**S-G4-202**	**S-73**
S-G4-38	**S-G4-86**	**S-G4-152**	**S-13**	**S-96**
S-G4-39	**S-G4-87**	**S-G4-153**	**S-14**	**S-97**
S-G4-40	**S-G4-88**	**S-G4-154**	**S-18**	**S-120**
S-G4-41	**S-G4-93**	**S-G4-155**	**S-19**	**S-121**
S-G4-42	**S-G4-94**	**S-G4-158**	**S-23**	**S-144**
S-G4-43	**S-G4-95**	**S-G4-159**	**S-36**	**S-145**
S-G4-44	**S-G4-98**	**S-G4-160**	**S-37**	**S-168**
S-G4-45	**S-G4-99**	**S-G4-161**	**S-60**	**S-169**
S-G4-46	**S-G4-100**	**S-G4-162**	**S-61**	**S-192**
S-G4-47	**S-G4-105**	**S-G4-163**	**S-84**	**S-193**
S-G4-50	**S-G4-106**	**S-G4-164**	**S-85**	**S-PAM-Cap102**
S-G4-51	**S-G4-107**	**S-G4-165**	**S-89**	**S-PAM-Cap103**
S-G4-52	**S-G4-110**	**S-G4-166**	**S-90**	**S-PAM-Cap104**
S-G4-53	**S-G4-111**	**S-G4-167**	**S-91**	**S-PAM-Cap113**
S-G4-54	**S-G4-112**	**S-G4-170**	**S-92**	**S-PAM-Cap114**
S-G4-55	**S-G4-117**	**S-G4-171**	**S-101**	**S-PAM-Cap115**
S-G4-56	**S-G4-118**	**S-G4-172**	**S-108**	
S-G4-57	**S-G4-119**	**S-G4-173**	**S-109**	
S-G4-58	**S-G4-122**	**S-G4-174**	**S-116**	
S-G4-59	**S-G4-123**	**S-G4-175**	**S-125**	
S-G4-62	**S-G4-124**	**S-G4-176**	**S-126**	
S-G4-63	**S-G4-129**	**S-G4-177**	**S-127**	
S-G4-64	**S-G4-130**	**S-G4-178**	**S-128**	
S-G4-65	**S-G4-131**	**S-G4-179**	**S-132**	

Group B: DNA origami G4 (DO G) (with S-PAM-cap-x and Apt-cap-x)

Reagents	Volume
TAE/Mg^{2+}/K^{+} buffer	44μl
S-G4-x	0.25ul × 136 = 34μl
S-x	0.25ul × 50 = 12.5μl
Apt-cap-x	0.25ul × 12 = 3μl
S-PAM-cap-x	0.25ul × 6 = 1.5μl
M13	5μl

S-G4-15	**S-G4-67**	**S-G4-136**	**S-G4-185**	**S-181**
S-G4-16	**S-G4-68**	**S-G4-137**	**S-G4-186**	**S-194**
S-G4-17	**S-G4-69**	**S-G4-138**	**S-G4-187**	**S-198**
S-G4-20	**S-G4-70**	**S-G4-139**	**S-G4-188**	**S-199**

S-G4-21	S-G4-71	S-G4-140	S-G4-189	S-203
S-G4-22	S-G4-74	S-G4-141	S-G4-190	S-204
S-G4-26	S-G4-75	S-G4-142	S-G4-191	S-24
S-G4-27	S-G4-76	S-G4-143	S-G4-195	S-25
S-G4-28	S-G4-77	S-G4-146	S-G4-196	S-48
S-G4-29	S-G4-78	S-G4-147	S-G4-197	S-49
S-G4-30	S-G4-79	S-G4-148	S-G4-200	S-72
S-G4-31	S-G4-80	S-G4-149	S-G4-201	S-73
S-G4-32	S-G4-81	S-G4-150	S-G4-202	S-96
S-G4-33	S-G4-82	S-G4-151	S-13	S-97
S-G4-34	S-G4-83	S-G4-152	S-14	S-120
S-G4-35	S-G4-86	S-G4-153	S-18	S-121
S-G4-38	S-G4-87	S-G4-154	S-19	S-144
S-G4-39	S-G4-88	S-G4-155	S-23	S-145
S-G4-40	S-G4-93	S-G4-158	S-36	S-168
S-G4-41	S-G4-94	S-G4-159	S-37	S-169
S-G4-42	S-G4-95	S-G4-160	S-60	S-192
S-G4-43	S-G4-98	S-G4-161	S-61	S-193
S-G4-44	S-G4-99	S-G4-162	S-84	S-PAM-Cap102
S-G4-45	S-G4-100	S-G4-163	S-85	S-PAM-Cap103
S-G4-46	S-G4-105	S-G4-164	S-89	S-PAM-Cap104
S-G4-47	S-G4-106	S-G4-165	S-90	S-PAM-Cap113
S-G4-50	S-G4-107	S-G4-166	S-91	S-PAM-Cap114
S-G4-51	S-G4-110	S-G4-167	S-92	S-PAM-Cap115
S-G4-52	S-G4-111	S-G4-170	S-101	Apt-Cap1
S-G4-53	S-G4-112	S-G4-171	S-108	Apt-Cap2
S-G4-54	S-G4-117	S-G4-172	S-109	Apt-Cap6
S-G4-55	S-G4-118	S-G4-173	S-116	Apt-Cap7
S-G4-56	S-G4-119	S-G4-174	S-125	Apt-Cap11
S-G4-57	S-G4-122	S-G4-175	S-126	Apt-Cap12
S-G4-58	S-G4-123	S-G4-176	S-127	Apt-Cap205
S-G4-59	S-G4-124	S-G4-177	S-128	Apt-Cap206
S-G4-62	S-G4-129	S-G4-178	S-132	Apt-Cap210
S-G4-63	S-G4-130	S-G4-179	S-133	Apt-Cap211
S-G4-64	S-G4-131	S-G4-182	S-156	Apt-Cap215
S-G4-65	S-G4-134	S-G4-183	S-157	Apt-Cap216
S-G4-66	S-G4-135	S-G4-184	S-180	

b. Mix the above reagents and briefly centrifuge them. The mix is first heated at 95 °C for 10 minutes. Subsequently, annealing is performed by slowly cooling the mixture from 95 °C to 20 °C at a rate of 1 °C/min.
c. The Amicon® Ultra-0.5 Centrifugal Filter Devices (100kDa) are used to remove redundant helper strands three times. The purified DO G

solution is obtained.

2. Loading functional strands
   a. Add 1.5 μL of the PAM-rich stock solution to group A. Add 1.5μl of the PAM-rich and aptamer stock solution to group B. Then, restore the volume to 100ul with TAE/Mg^{2+}/K^{+}buffer.
   b. Mix the above reagents and briefly centrifuge them. The mix is annealed from 45 °C to 25 °C at a rate of 5 min/°C for six cycles.
   c. The Amicon® Ultra-0.5 Centrifugal Filter Devices (100kDa) are used to remove redundant PAM-rich and aptamer three times. The purified $DO_{PAM}G$ and $DO^{A}_{PAM}G$ solution are obtained.

3. Loading of hemin
   a. Add 1 μL of hemin stock solution to the purified $DO_{PAM}G$ and $DO^{A}_{PAM}G$ solution obtained in the last step. Then, restore the volume to 100ul with TAE/Mg^{2+}/K^{+}buffer.
   b. Gently resuspend the solution by pipetting three times using a wide-bore pipette tip.
   c. Briefly centrifuge at 500 × g for 3 seconds to sediment droplets.
   d. Incubate at room temperature for 1 hour.
   e. The Amicon® Ultra-0.5 Centrifugal Filter Devices (100 kDa) are used to remove redundant hemin. The purified $DO_{PAM}GH$ and $DO^{A}_{PAM}GH$ solution are obtained. Then, restore the volume to 100ul with TAE/Mg^{2+}/K^{+}buffer.

4. Repeat the previous steps for two times.

5. Preparation of NPN stock solution:
   a. Weigh 0.0219g NPN powder using an analytic balanced.
   b. Transfer the weighed powder into 10mL anhydrous ethanol and mix the solution thoroughly. Label the centrifuge tube as NPN-1.
   c. Pipette 1mL NPN-1 into 9mL anhydrous ethanol and mix the solution thoroughly. Label the centrifuge tube as NPN-2.
   d. Pipette 1mL NPN-2 into 19mL PBS and mix the solution thoroughly. Label the centrifuge tube as NPN-3.
   e. Pipette 1mL NPN-2 into 19mL 0.067M Tris-HCl and mix the solution thoroughly. Label the centrifuge tube as NPN-4.
   f. Pipette 1mL NPN-2 into 1mL PBS and mix the solution thoroughly. Label the centrifuge tube as NPN-5.

6. Preparation of EDTA Na_2 solution:
   a. Weigh 0.186g EDTA Na_2.
   b. Add 100mL ddH_2O to solubilize the weighed EDTA Na_2.

7. Dilution of bacterial suspension:
   a. Take 5mL of overnight cultured *E. coli* ATCC25922 suspension and add it into five 1.5mL centrifuge tubes. Centrifuge the five 1.5mL centrifuge tubes at 4000 rpm for 3 minutes.
   b. Discard the supernatant and resuspended the bacterial sediment in each 1.5mL centrifuge tube with 1mL PBS.
   c. Pipette 4mL PBS into a 15mL centrifuge tube.
   d. Add aliquots of bacterial suspension into the 15mL centrifuge tube until the OD600 value approaches 1.5.

8. Preparation of the components of each group:
   a. Prepare ten 1.5mL centrifuge tubes and label them from 1 to 10.
   b. For tubes 7-10, add 150uL of bacterial suspension. Centrifuge the four tubes at 12,000 rpm for 3 minutes. Discard the supernatant in each tube. Resuspend the bacterial sediments in tubes 7 and 9 with 28.8μL PBS and resuspend the bacterial sediments in tubes 8 and 10 with 22.8μL PBS.
   c. Add other components of each group to the corresponding centrifuge tubes according to the table below.

Buffer	1	300μL PBS
	2	240μL PBS
		60μL 50μM NPN-3
Buffer + Cells	3	150μL PBS
		150μL PBS-diluted bacterial suspension
	4	90μL PBS
		60μL 50μM NPN-3
		150μL PBS-diluted bacterial suspension
EDTA	5	90μL 0.067M Tris-HCl
		60μL 5mM EDTA Na_2
		150μL PBS-diluted bacterial suspension
	6	30μL 0.2M Tris-HCl
		60μL 5mM EDTA Na_2
		60μL 50μM NPN-4
		150μL PBS-diluted bacterial suspension
$DO_{PAM}GH$	7	267μL 5nM DNA origami
		3μL 255μM Hemin
		1.2μL 12.5mM H_2O_2
	8	267μL 5nM DNA origami
		3μL 255μM Hemin
		1.2μL 12.5mM H_2O_2
		6μL 500μM NPN-5
$DO^{A}_{PAM}GH$	9	267μL 5nM aptamer-loaded DNA origami

		3μL 255μM Hemin
		1.2μL 12.5mM H_2O_2
	10	267μL 5nM aptamer-loaded DNA origami
		3μL 255μM Hemin
		1.2μL 12.5mM H_2O_2
		6μL 500μM NPN-5

9. Measurements of fluorescent intensity:
   a. Transfer the solution in the centrifuge tubes into the black-bottomed 96-well plate. Each group corresponds to three wells on the plate. Incubate the plate at room temperature for 3 minutes.
   b. Place the black-bottomed 96-well plate into SpectraMax® iD5 Microplate Reader and select “Endpoint” mode under the fluorescence module.
   c. Set the excitation length to 350nm and the emission length to 420nm.
   d. Set PMT gain to 500.
   e. Record the relative fluorescent unit of each well.