Electronic Supplementary Material (ESI) for Analytical Methods.

A Novel Ratiometric Fluorescence Probe From Hemicyanine Derivate For Detecting NAD(P)H In Cell Microenvironment

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Contents

1. Absorption titration experiment of Cou-In

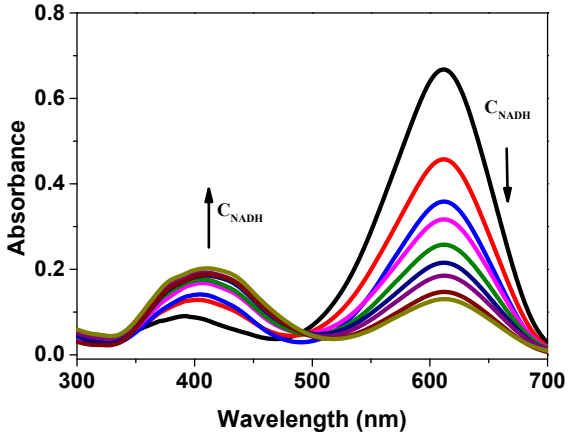

Figure S1. Absorption spectra of 10 μM Cou-In treated with various amounts of NADH.

2. The fluorescence spectra of Cou-In responding to NADH

The steady-state fluorescence emission spectra of NADH and Cou-In in the absence and presence of NADH were shown in Figure S2. It can be noted from Figure S2 that the fluorescence emission spectra of Cou-In treated with NADH were enhanced significantly at 550 nm , whereas the emission at 675 nm decreased distinctly.

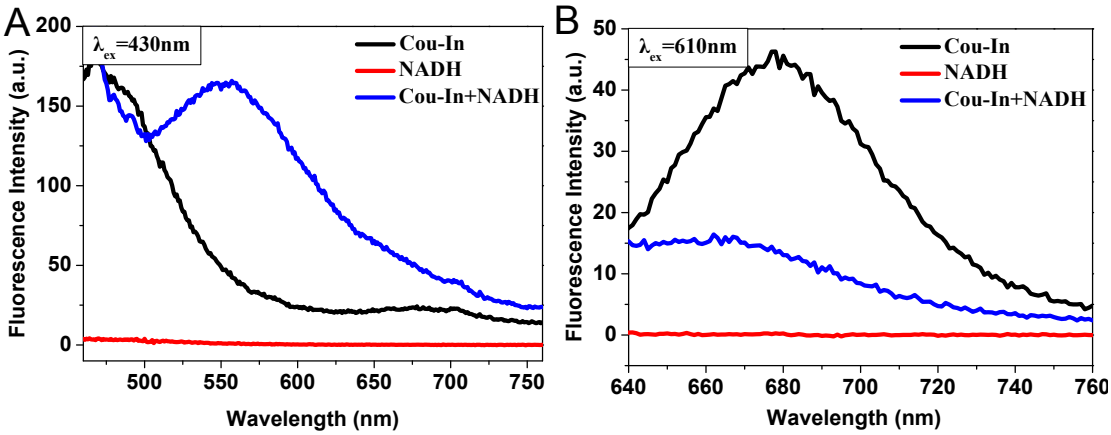

Figure S2. The fluorescence emission spectra (A-λ_{ex}=430 nm; B-λ_{ex}=610 nm) of NADH (50.0 μM) and Cou-In (10.0 μM) in the absence and presence of NADH.

3. Fluorescence titration experiment of Cou-In

The fluorescence titration experiment of Cou-In upon the addition of NADH with different concentrations were performed. The relationship of ratio (I_{550}/I_{675}) and the concentrations of NADH was shown in Figure S3A, and the linearity curve between the ratio and concentrations (0-25 μM) was shown in Figure S3B.

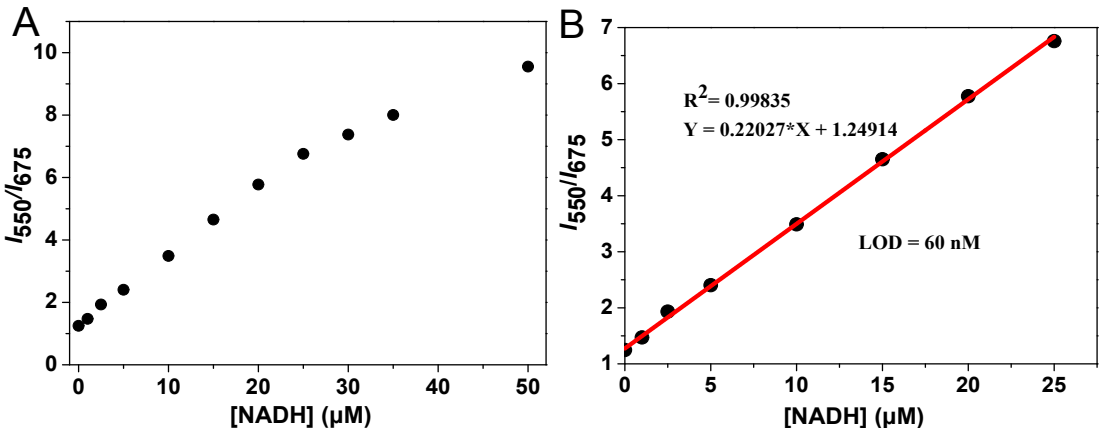

Figure S3. (A) The changes in fluorescence ratio (I_{550}/I_{675}) of Cou-In (10.0 μM) upon the addition of various concentrations of NADH: (0, 1.0, 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, 30.0, 35.0, 50.0 μM).

(B) The linearity curve between the fluorescence ratio (I_{550}/I_{675}) and the concentrations of NADH (0-25.0 μM).

4. Determination of the limit of detection

The limit of detection was calculated based on the method used in the previous literature [1]. The fluorescence ratio (I_{550}/I_{675}) of Cou-In was measured by eleven times and the standard deviation of blank measurement was obtained. The fluorescence ratio (I_{550}/I_{675}) was plotted as a concentration of NADH. The limit of detection was calculated with the following equation:

$$\text{Limit of detection (LOD)} = 3\sigma/k$$

Where σ is the standard deviation of blank measurement, k is the slope between the fluorescence ratio (I_{550}/I_{675}) versus the NADH concentration.

5. The cytotoxicity (HeLa cells) of Cou-In

The cytotoxicity (HeLa cells) of Cou-In was tested by CCK8 methods. The experiment was divided into control group and experimental group. The control group was added with 100 uL/well complete medium. Diluted Cou-In to 2%, 4%, 6%, 8%, 10% working solution, then added 100 uL/well, respectively. Three holes were set for each concentration.

HeLa cells in logarithmic growth phase were taken for cell counting and cell concentration was adjusted. According to the sample, cells were co-cultured with the

cells for 24 h. The number of cells in each well was 4×10^3, and the cells were spread into 96-well plates. Cultured overnight in a 37 ℃ incubator with 5% CO_2. According to the above group processing and training 24 h. Removed the medium. Clean the wells three times with PBS, then add medium containing 10% CCK-8, 5% CO_2 and culture in an incubator at 37℃ for 2 hours. The absorbance value at 450 nm was detected by enzyme plate analyzer.

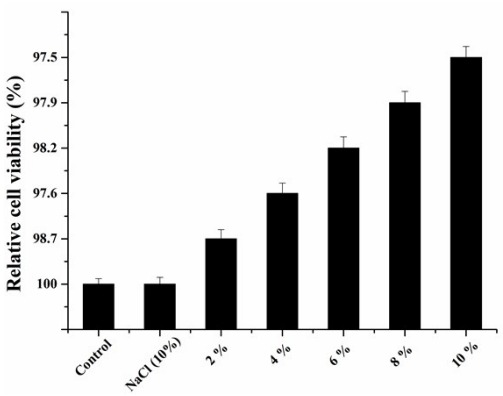

Figure S4. The cytotoxicity (HeLa cells) of Cou-In was tested by CCK8 method

6. Effect of Cou-In on the activity of ADH

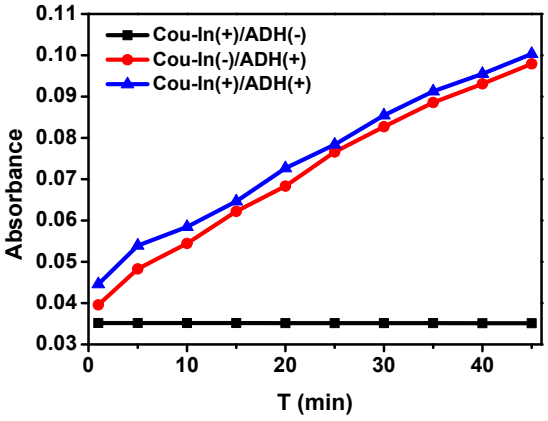

Figure S5. Monitoring ADH activity by detecting increase in NADH absorption at 340nm in the presence of 10 μM Cou-In (blue line), 50 μM NADH, 2% ethanol, and 2 U/mL ADH were

incubated at room temperature. The red line and black line represent no Cou-In and ADH, respectively. The apparent difference of residual absorbance at the end point between Cou-In (+) and (-) is due to the absorption of Cou-In itself.

7. Effects of ethanol and NAD^+ on the absorption spectra of Cou-In

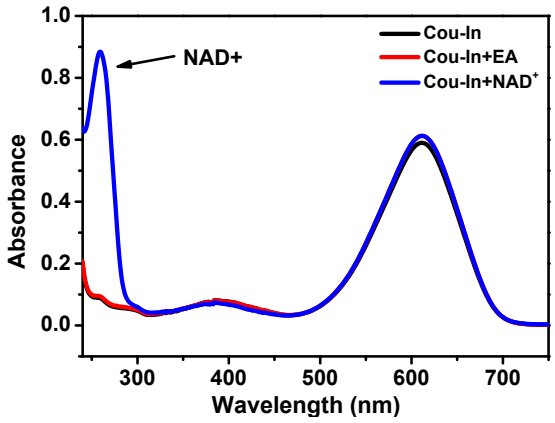

Figure S6. Absorption spectra of 10.0 μM Cou-In in the absence of ethanol and NAD^+ (black line). The red line and blue line represent in the presence of ethanol or NAD^+.

8. Detection of ADH enzyme activity by absorbance

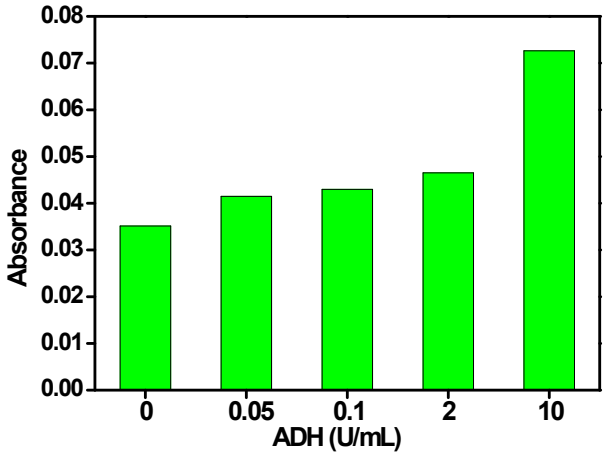

Figure S7. The absorption value of NADH at 340nm varies with the concentration of ADH.

9. Additional Spectroscopic Data

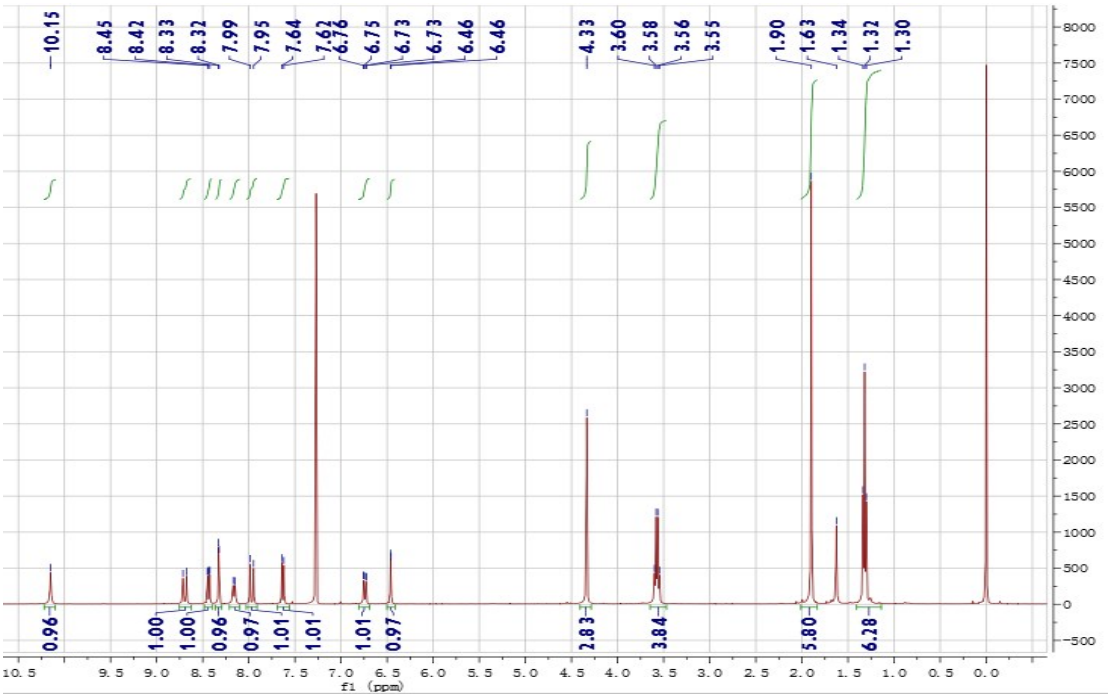

Figure S8. ^{1}H NMR of the compound Cou-In

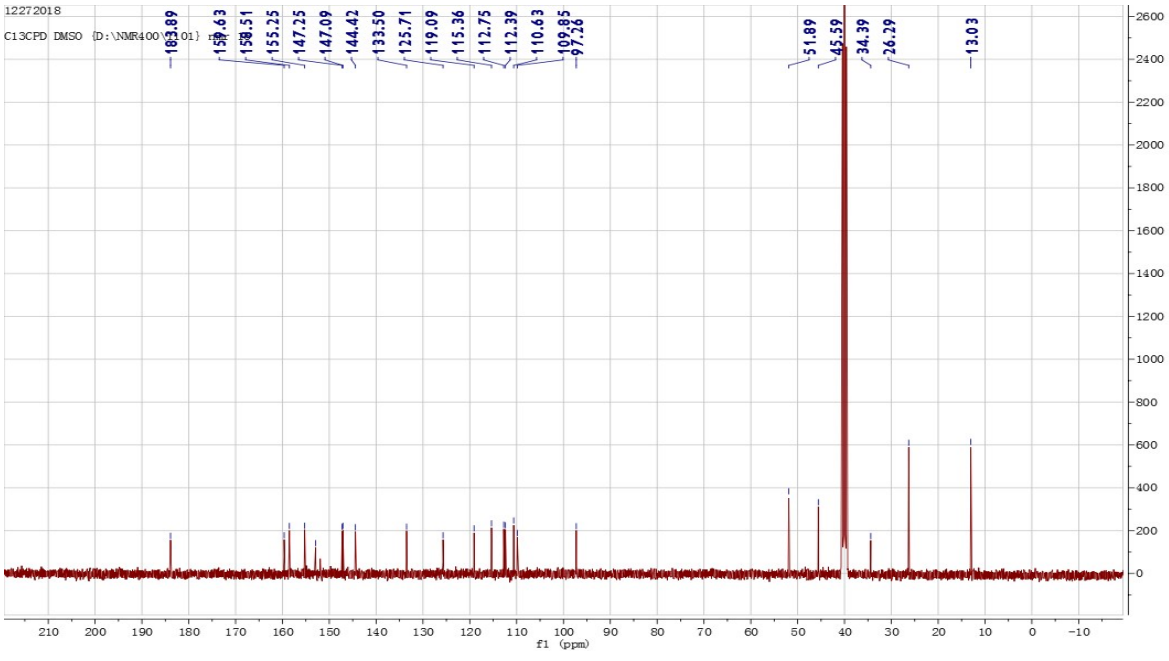

Figure S9. ^{13}C NMR of the compound Cou-In

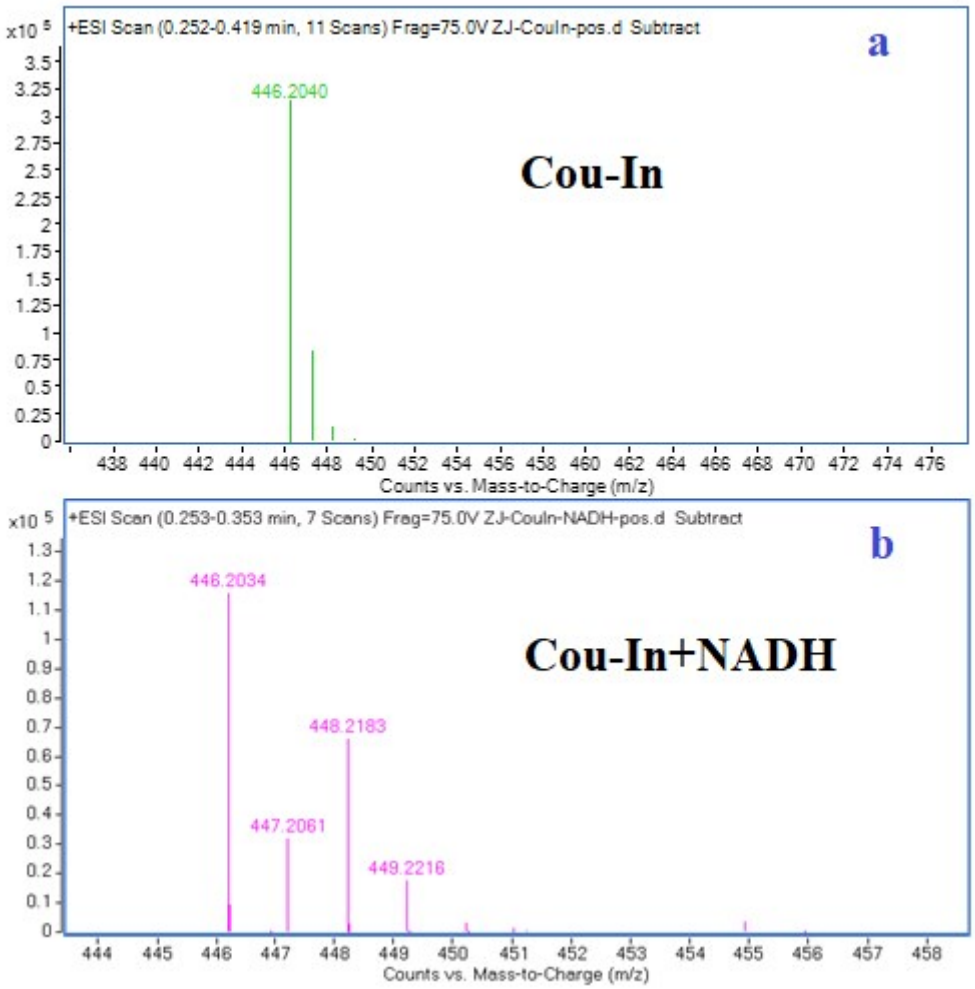

Figure S10. **a.** HRMS(API-ES) of the compound Cou-In, **b.** HRMS(API-ES) of the compound Cou-In+NADH

10. References

[1] B. Zhu, C. Gao, Y. Zhao, *et. al.* A 4-hydroxynaphthalimide-derived ratiometric fluorescent chemodosimeter for imaging palladium in living cells, Chem. Commun. 47 (2011) 8656-8658