Electronic Supplementary Information (ESI)

In situ semi-quantitative assessment of single cell viability by resonance Raman spectroscopy

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EXPERIMENTAL SECTION

Reagents

MTT salts, also called Thiazolyl Blue Tetrazolium Bromide (3-[4,5-Dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide), and MTT-formazan (1-[4,5-Dimethylthiazol-2-yl]-3,5-diphenylformazan) were obtained from Sigma-Aldrich. DMSO (Dimethyl sulfoxide) and all other chemicals were obtained from Beijing Chemical Reagent Factory.

The phosphate-buffered saline (PBS; 0.01 M, pH 7.2) used in this study contained 0.8% NaCl, 0.02% KH_2PO_4, 0.02% KCl and 0.12% $Na_2HPO_4 \cdot 12H_2O$. All chemicals were analytical-grade reagents and used without further purification. Mill-Q water was used in the study.

Instruments and measurement

The UV-vis spectra were recorded on a Shimadzu UV-3600 spectrophotometer. The fluorescence spectra were measured with RF-5301PC spectrofluorophotometer (Shimadzu, Japan) equipped with 1.0 cm quartz cells. The widths of the excitation slit and the emission slit were set to 5 nm and 3 nm, respectively. An excitation wavelength of 325 nm was chosen and the emission wavelength range was 350-630 nm.

Raman spectra were recorded on a LabRam Aramis Raman Microscope system (Horiba-JobinYvon) equipped with a multichannel air-cooled charge-coupled device (CCD) detector. Spectra were excited using the ultraviolet (UV) at 325 nm excitation wavelength and the 633nm line of a HeNe narrow bandwidth laser (Melles Griot). The 514 nm excitation lines were collected at room temperature with the Raman spectrometer (T64000, Horiba-JobinYvon) equipped with a charge coupled device (CCD) detector.

The length of acquisition time is 30s (10s × 3 windows) for a single RRS spectrum with an output laser of 1.7 mW excitation at 633 nm (100μm hole, 1800 Grating lines/mm resolution spatial and objective 10×). The samples were observed with objective 10× (NA=0.25) lense which give laser spots with a diameter of 3μm, respectively. Since formazan compounds are photo-sensitive to laser irradiation, the provided set of five density filters was used to modulate the laser power on the samples with both of 633nm and 514nm.

Micro-Raman spectroscopy was recorded using a Horiba–Jobin Yvon ARAMIS Raman spectrometer with a HeNe laser 633 nm, 1.7 mW, 100μm hole, 1800 Grating lines/mm resolution spatial and 10s acquisition time (single window); the analyzed areas were pinpointed by using the video camera attached to the microscope. The LabRAM Aramis micro-Raman is furthermore equipped with two front illuminated Charge-Coupled Device (CCD) detectors, both cooled at 70 ℃. Acquisition and basic treatment of spectra were performed with LabSpec 5 software (Jobin Yvon-Horiba) and OriginPro 8.0: baseline was subtracted, and slight smoothing operation was done in the recorded spectra.

Preparation of MTT and formazan stock solution and cells

5 mg/mL MTT stock solution was prepared by dissolving MTT salts in phosphate-buffered saline (PBS) buffer (pH=7.2); it was filtered and ready for use at 4 °C. MTT formazan stock solution was prepared by dissolving formazan (4 mg) in DMSO (4 mL) at room temperature. The human lung cancer cell line A549 and rat smooth muscle cells were obtained from Department of Anatomy (School of Basic Medical Sciences, Jilin University, P.R.China).

Fig. S1

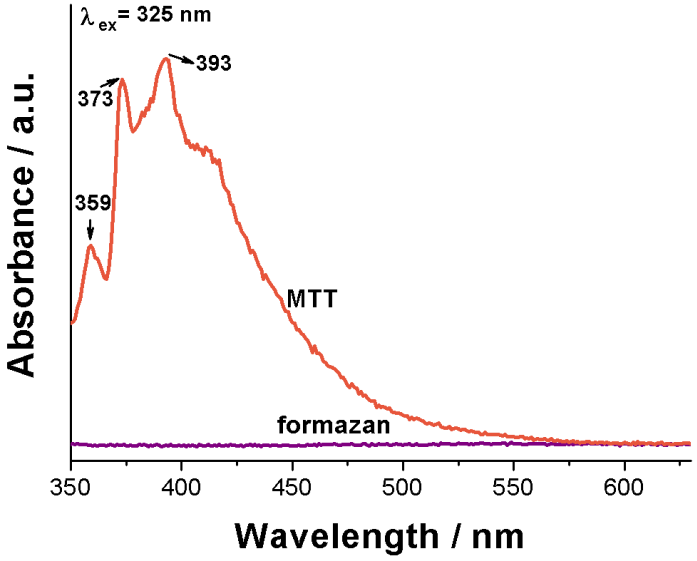

Fig. S1 a) Fluorescence spectra of the 1 mg/mL MTT and 0.01mg/mL formazan with 325 nm excitation wavelength.

Fig. S2

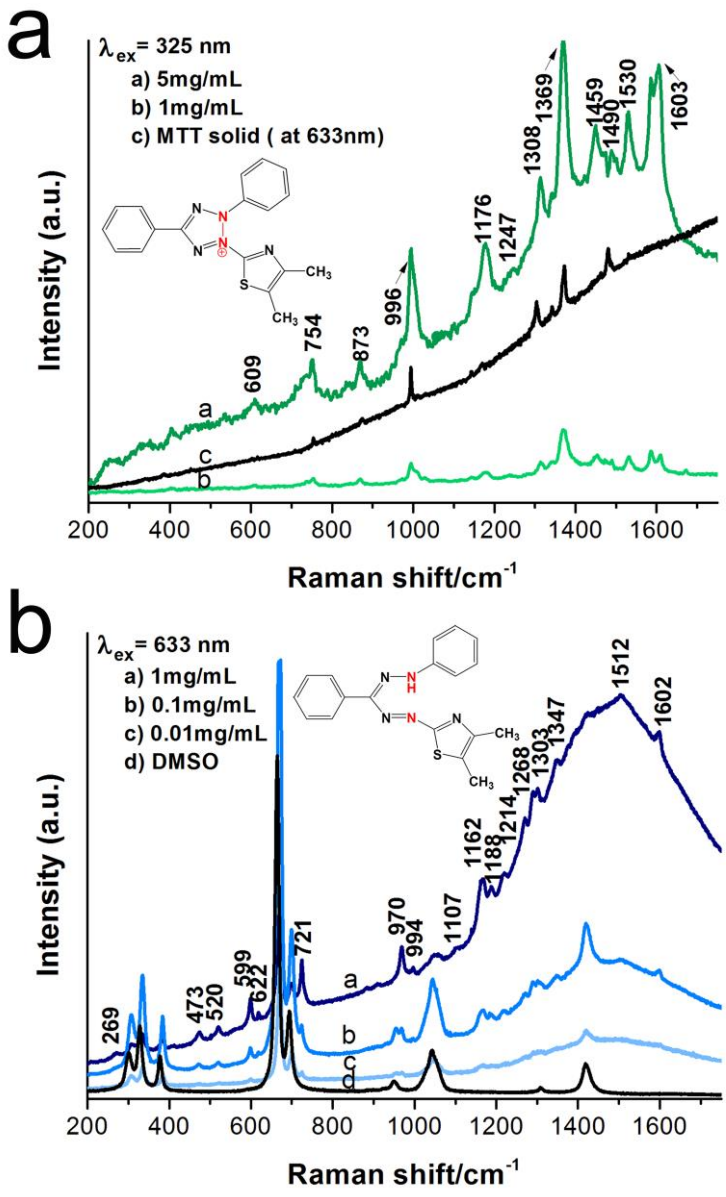

Fig. S2 a) Structure of MTT (the inset) and resonant Raman spectra of MTT-PBS solution with 325 excitation and normal Raman spectra of MTT solid with 633 nm excitation, b) Structure of formazan (the inset) and resonant Raman spectra of formazan-DMSO solution with 633 nm excitation.

Fig. S3

In Fig.3(a) the intensity of absorption at 514nm is indeed higher than the intensity of absorption at 633nm. But the experiment results are always show the reverse that the signal of formazan RRS measured with 633nm excitation is much higher than measured with 514nm excitation when spectra are collected with same measurement conditions. This phenomenon would be interpreted in detail as below:

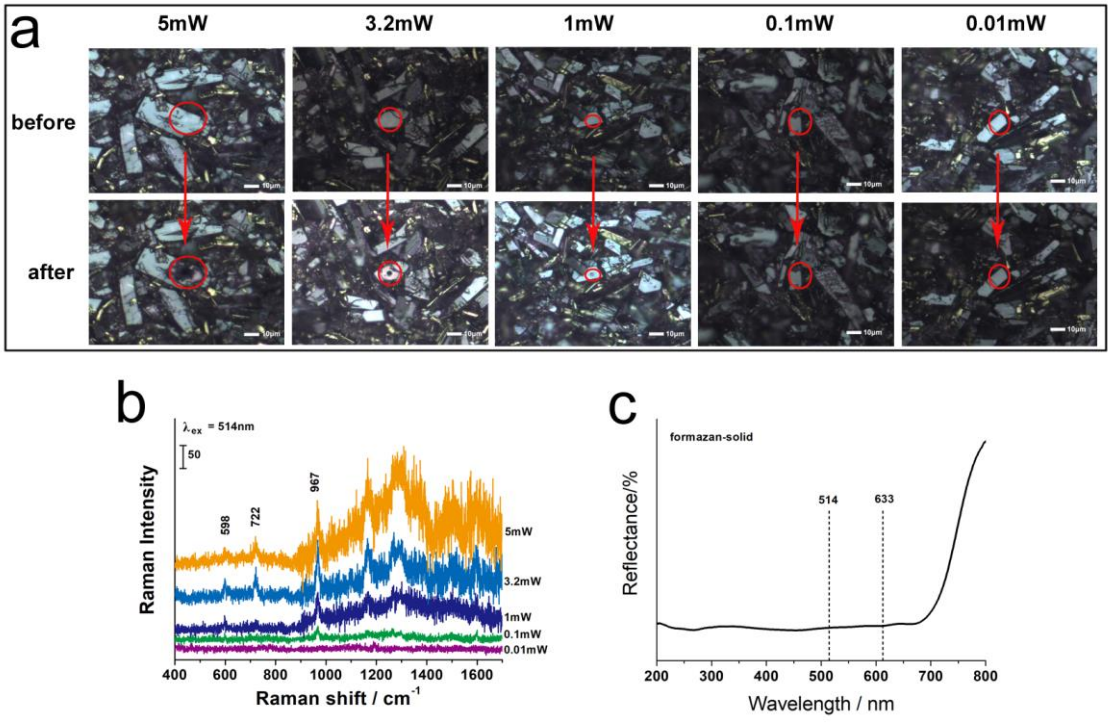

Fig. S3 (a) The photograph of pure formazan solid (before and after power dependence RRS measurement); (b) Acquisition power-dependence of formazan solid with 514nm excitation (10×object, 30s per spectrum, 100μm hole, 1800 Grating lines/mm). The laser was off during each time interval. The height difference between formazan peaks (eg. 598cm^{-1}, 722 cm^{-1} and 967 cm^{-1}) and cell plate peaks (eg. 618 cm^{-1} and 1000 cm^{-1}) shown that the optical laser output power is 0.1mW. (c) UV-vis diffuse reflection spectra of the formazan solid.

1) For Formazan solids, the absorption intensities at 514 nm and 633 nm are equal.

Notice that the absorption spectra in Fig.2(a) is measured from a solution of formazan. Besides, we measured the UV-Vis diffuse reflection spectrum of formazan solid. The results show that the absorption spectra of the formazan in the solution and solid state are different, see Fig.S3(c). Therefore, when we measured the formazan solid in single cell, the intensity measured with two excitations should be the same. But why does the RRS measurement

with 633nm excitation (Fig.S5) is much stronger than with 514nm in actual measurement? The emergence of this phenomenon is due to another reason, see reason 2).

2) The 514 nm wavelength laser with higher energy can more easily decompose the sample than the 633 nm wavelength laser, as shown in Fig.S3(a).

A comparison of the solid samples before and after the Raman test showed that the samples had been decomposed under high laser power conditions. As the power of the laser increases, the decomposition of the sample becomes more severe. Therefore, the results shown that the RRS measurement with 633nm excitation (Fig.S5) is much stronger than with 514nm (Fig.S3) in actual measurement. And the 633 laser is the most suitable excitation for this method.

Fig. S4 and Table S1

The time of RRS signal acquisition is very important for the RRS signal detection. Since formazan compounds are photo-sensitive to laser irradiation, we provided set of five density filters which was used to modulate the laser power on the samples with 633nm and 514nm, respectively. In this work, the optimal time of RRS signal acquisition is three accumulations of 10 s (30s per spectrum), which can help to obtain a strong formazan RRS signal within a single cell and prevent being photo-decomposing. The instrument conditions were: 100μm confocal hole, 1800 lines/mm grating, and 10× objective Olympus lens (N.A.=0.25, WD=10.6 mm).

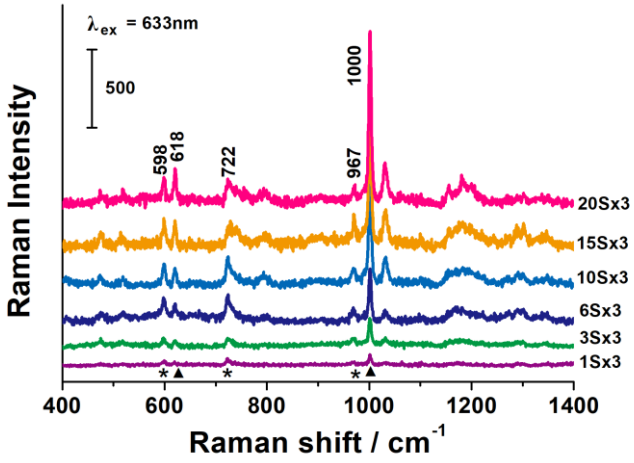

Fig. S4 Acquisition time-dependence of formazan in a single rat smooth muscle cell with 633nm excitation (10×object, 1.7 mW, 100μm hole, 1800 Grating lines/mm). The laser was off during each time interval. The height difference between formazan peaks (eg. 598cm^{-1}, 722 cm^{-1} and 967 cm^{-1}) and cell plate peaks (eg. 618 cm^{-1} and 1000 cm^{-1}) shown that the optical acquisition time is 30s per spectrum (10s×3).

Table S1. Calculation of the I_{722} of acquisition time dependent corresponding Fig. S4 and the optical acquisition time.

Acquisition time	I_{722}	photo-decomposing	the optical time
20s×3	w	yes	
15s×3	w	yes	
10s×3	vs	no	√
6s×3	s	no	
3s×3	w	no	
1s×3	vw	no	

** 1."vs" stand for very strong intensity, "s" stand for strong intensity, "w" stand for weak intensity, "vw" stand for very weak intensity.2.The RRS spectra were carefully baselined and normalized by the I_{1000} before calculation.*

Fig. S5 and Table S2

In this work we found that formazan compound is photo-sensitive to laser irradiation. However, when the RRS spectrum of formazan solid was collected under the same conditions, the degree of decomposition with 633 nm and 514 nm excitations were different. It is easier to be decomposed with 514nm laser. Therefore, we chose 633 laserline as the excitation of RRS in this work. Hence, the provided set of five density filters was used to modulate the laser power on the samples. The power-dependent of RRS scattering are presented. The results shown that the optimal condition of RRS measurement is measured with laser output power of 1.7mW at 633nm excitation

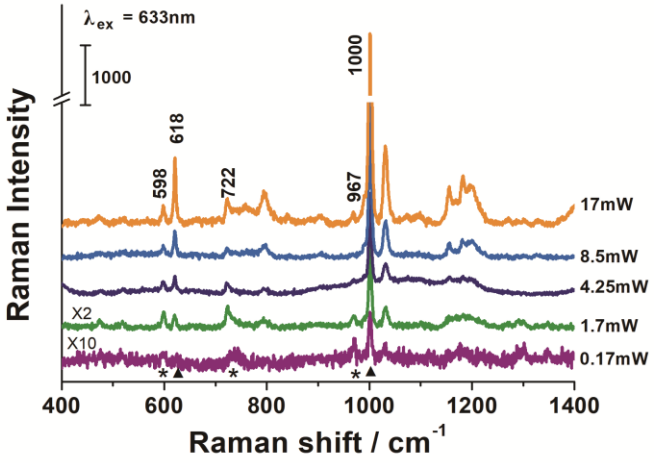

Fig.S5 Acquisition power-dependence of formazan in a single rat smooth muscle cell with 633nm excitation (10×object, 30s per spectrum, 100μm hole, 1800 Grating lines/mm). The laser was off during each time interval. The height difference between formazan peaks (eg. 598cm^{-1}, 722 cm^{-1} and 967 cm^{-1}) and cell plate peaks (eg. 618 cm^{-1} and 1000 cm^{-1}) shown that the optical laser output power is 1.7mW.

Table S2. Calculation of the I_{722} of power dependent corresponding Fig. S5 and the optical acquisition time.

Laser output power	I_{722}	photo-decomposing	the optical power
17mW	w	yes	
8.5mW	w	yes	
4.25mW	s	yes	
1.7mW	vs	no	√
0.17mW	vw	no	

** 1."vs" stand for very strong intensity, "s" stand for strong intensity, "w" stand for weak intensity, "vw" stand for very weak intensity.2.The RRS spectra were carefully baselined and normalized by the I_{1000} before calculation.*

Fig. S6

It is shown that the RRS intensities of 722 cm^{-1} and 967 cm^{-1} were positively correlated with the formazan concentration. RRS intensities of 722 cm^{-1} and 967 cm^{-1} are significantly increased with an increase in the concentration of formazan. The intensity of the band at 722 cm^{-1} and 967 cm^{-1} was found to be linear with the value of formazan concentration within the concentration range from 0.06mg/mL to 0.001 mg/mL.

In conclusion, the concentration dependence curves (d) shown that when the RRS intensity of peak 722 cm^{-1} is higher than 350, the detected formazan amount is more than 0.002 mg in a single cell. Also, the concentration dependence curves (e) shown that when the RRS intensity of peak 967 cm^{-1} is higher than 320, the detected formazan amount is more than 0.002 mg in a single cell.

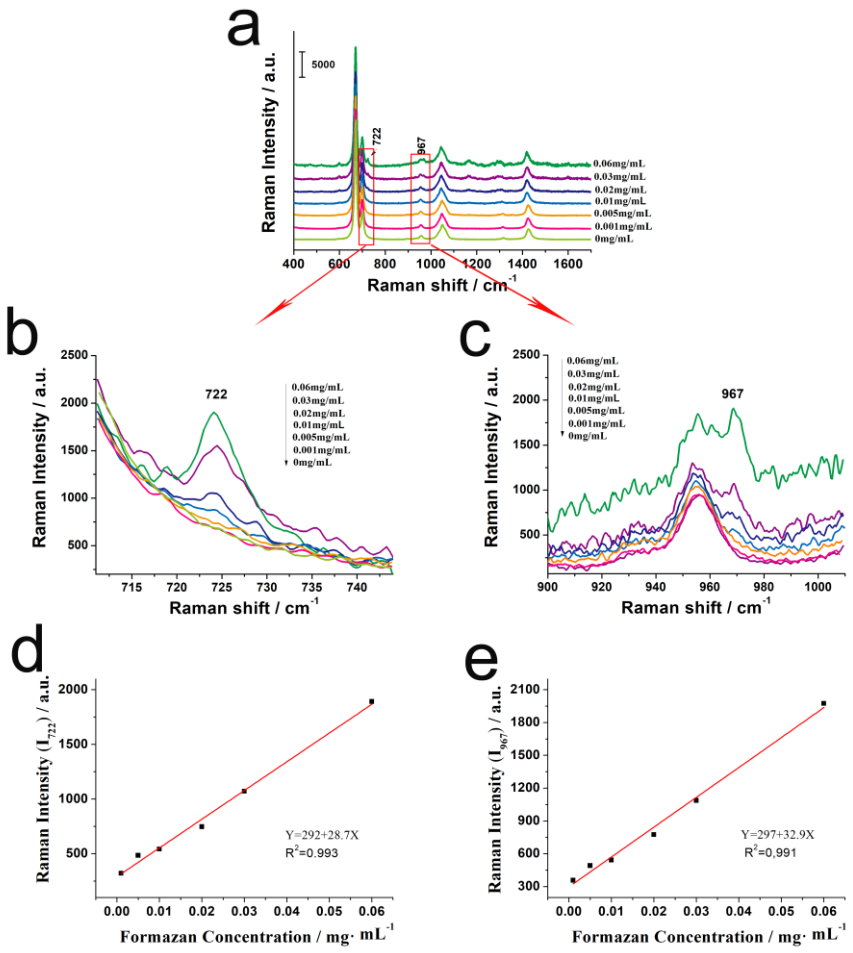

Fig.S6 (a) Concentration-dependent RRS spectra of formazan solution. The excitation wavelength is 633 nm; (b-c) the amplifying RRS in (a); Intensity ratio of RRS peak at 722 cm^{-1} and 967 cm^{-1} versus the concentration of formazan solution.

Fig. S7

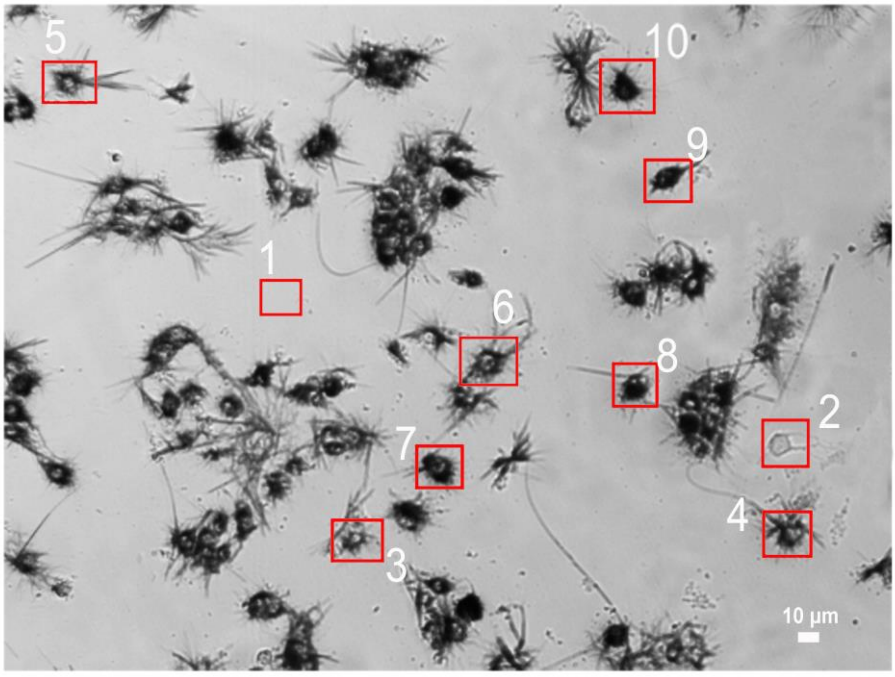

Fig. S7 photograph show image of the A549 cells after incubation with the MTT solution. note “hairy” extensions representing long-shaped formazan crystals.

Fig. S8

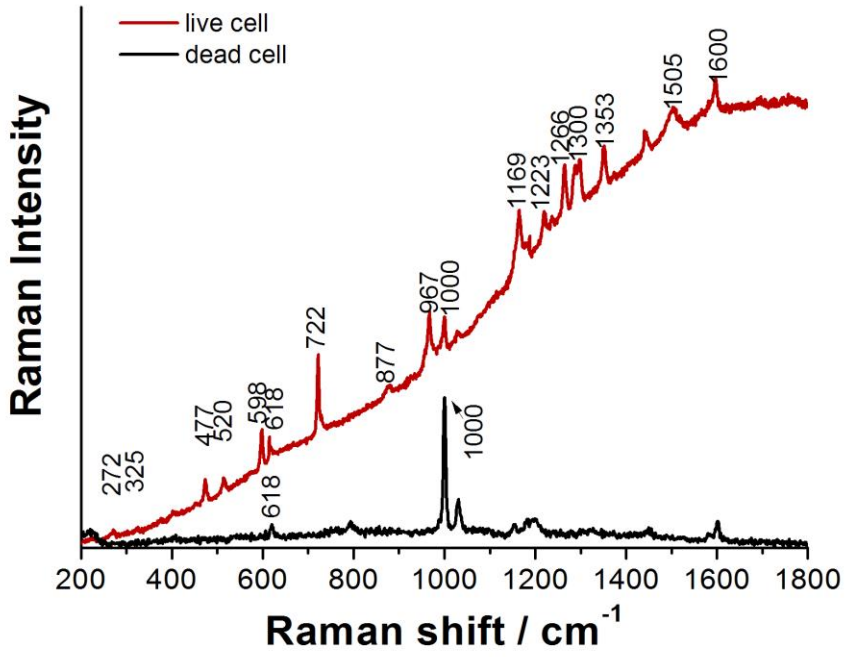

Fig. S8 Raman spectra observed from the MTT assay of A549 live cell (in red) and dead cell (in black). The excitation wavelength is 633 nm.

Fig. S9

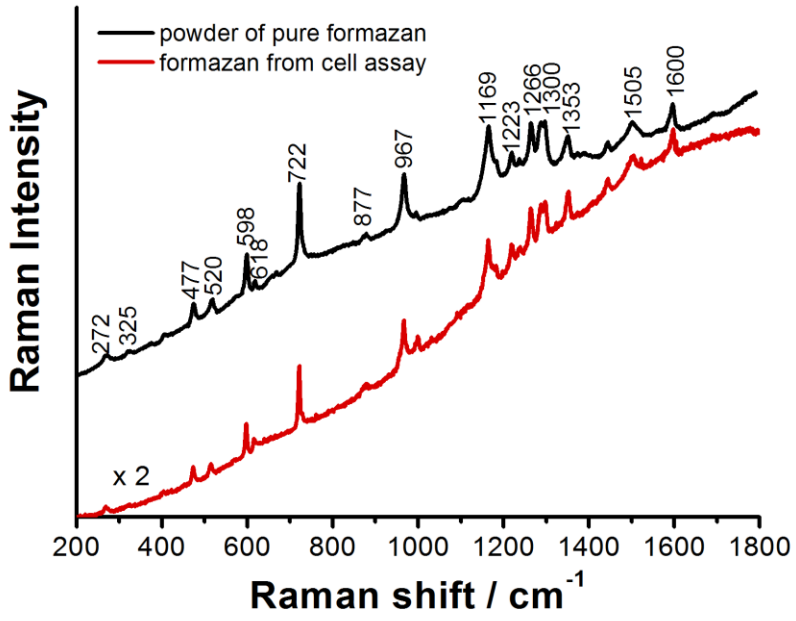

Fig. S9 Raman spectra of formazan observed from Cell MTT assay with A549 cells (in red), (b) formazan solid (in black). The excitation wavelength is 633 nm.

Fig. S10

The object we used is 10× with N.A.=0.25. We calculate the laser spot size as follow:

Laser spot size (633nm) =1.22λ / N.A.=1.22×633nm / 0.25=3.1μm

Laser spot size (514nm) =1.22λ / N.A.=1.22×514nm / 0.25=2.5μm

Therefore, in this work the size of laser spot is about 3μm with 633nm excitation and 2.5 μm with 514nm excitation. The size of A549 cell and rat smooth muscle cell which we used in this work is about 10μm. Thus, cell size is larger than beam size. In the RRS measurements, we focus the laser beam near the center of a single cell.

To assess whether this RRS method can be used for the quantitative analysis of formazan in single cell, we repeated the RRS test in the same cell and calculated the I_{967}/I_{1000} ratio for each spectrum of one cell. Using rat smooth muscle cells, we measured in situ RRS spectra of formazan per cell and collected 5 points in the center part of one cell.

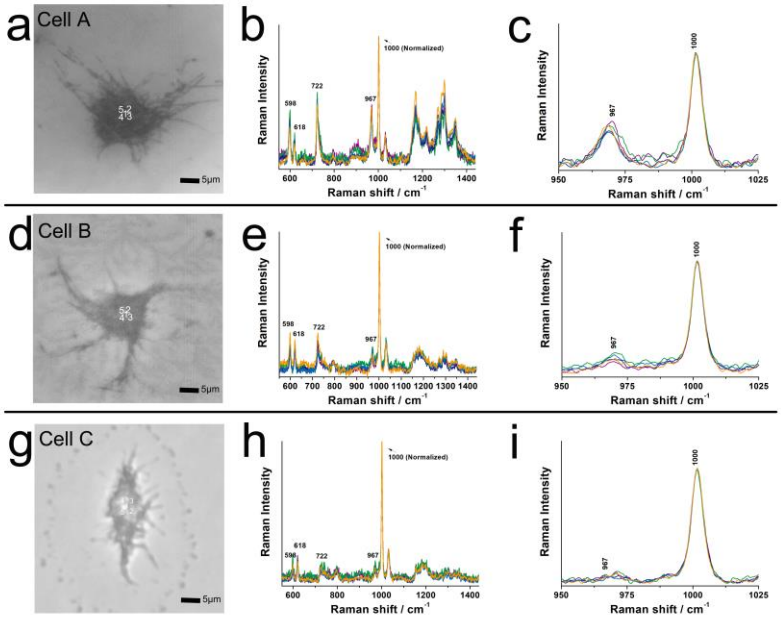

Fig. S10 In situ RRS spectra of formazan measured in three rat smooth muscle cells, which are numbered as “Cell A”, “Cell B” and “Cell C” (excitation: 633 nm, 10× object, laser power output: 1.7mW, grating: 1800 lines /mm, acquisition time: 30s per spectrum). The laser spot focus on five point (point 1-5 per cell) which in the center part of cell. (a) photograph of cell A; (b) profile of RRS and (c) the amplifying RRS correspond the point number in (a). (d) photograph of cell A; (e) profile of RRS and (f) the amplifying RRS correspond the point number in (d). (g) photograph of cell A; (h) profile of RRS and (i) the amplifying RRS correspond the point number in (g). The laser was off during each time interval. The Profile of relative Raman intensity at I_{967}/I_{1000} are shown in table S3.

Table S3

Table S3. Calculation of the ratio I_{967}/I_{1000} and semi-quantitation of the viability in a single cell analysis corresponding Fig. S10.

Cell No.	Point No.	Ratio(I_{967}/I_{1000})	Mean±SD	Viability	RSD
Cell A	1	0.3213	0.3199±0.0233	32%	7.3%
	2	0.3370			
	3	0.2975			
	4	0.2910			
	5	0.3528			
Cell B	1	0.0785	0.0852±0.0069	9%	8.1%
	2	0.0908			
	3	0.0915			
	4	0.0898			
	5	0.0753			
Cell C	1	0.0573	0.0575±0.0072	6%	12.6%
	2	0.0578			
	3	0.0645			
	4	0.0443			
	5	0.0635			