SUPPLEMENTARY MATERIALS AND METHODS

An optimized spectrophotometric assay for convenient and accurate quantitation of intracellular iron oxide nanoparticles

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Detailed protocol for ferene-s assay:

REAGENTS:
Glacial acetic acid (Fisher cat. No. A38-212)
Ammonium acetate (Fisher cat. No. A637-500, FW = 77.08)
Ferene-s (3-(2-Pyridyl)-5,6-di(2-furyl)-1,2,4-triazine-5′,5″-disulfonic acid disodium salt, Sigma cat. No P4272, FW = 494.37)
L-ascorbic acid (Sigma cat. No. A92902-100, FW =176.12)
TraceCERT Iron Standard for ICP (Sigma-Aldrich 43149-100ML, 1000±2 mg/l)
Nitric acid (Sigma cat. No. 30709-1L, ≥ 65%)
Standard 96 well plates (Thermo Fisher Scientific cat. No. 130188)
Phosphate buffered saline (PBS, Corning Cellgro cat. No. 21-040-CV)

EQUIPMENT:
Plate reader (SpectraMax-M5, Molecular Devices)
Flow hood (hazardous material)
Microcentrifuge
PH meter
SealRight® 1.5 ml tubes
Heat block

REAGENT PREPERATION:
Stock of (2 M) acetate buffer (pH 4.0 - 4.5) store at room temperature (stable for several months): Weigh 46.3 g of ammonium acetate, add 120 ml of glacial acetic acid and deionized water to final volume of 300 ml (measured pH ~ 4.3).

Stock of ferene-s (0.5 M) store in aliquots at 4º C protected from light (stable for several months): Weigh 0.5 g of ferene-s, add deionized water to 2.0 ml.

5x working buffer, 1 M l-ascorbic acid in 2 M acetate buffer (make fresh, stable for at least a week at 4º C): Weigh 2.0 g of l-ascorbic acid, add 2 M acetate buffer to 11.0 ml (enough for ~ 55 samples).

1x working solution: 10.0 ml of 5x working buffer, 0.5 ml of ferene-s stock, 39.5 ml of deionized water to final volume of 50.0 ml (stable for at least a week at room temperature).
10 N NaOH: Weigh 20.0 g of sodium hydroxide; add deionized water to final volume of 50 ml.

100 µg/ml Fe standards: Stock of Fe standards (1 mg/ml) are stored at 4º C in 5 ml aliquots sealed with parafilm. Dilute the stock solution in deionized water (add 100 µl of stock to 900 µl of water store at 4º C).

SIMPLIFIED PROCEDURE

Working solution (950 µl) can be directly added to each sample (50 µl) containing nanoparticles (including cells) in a 1.5 ml tube (without any further processing). After through mixing the samples are left at room temperature in the dark for ≥20 hrs. The absorbance is then measured at 595 nm in a flat bottom standard 96-well plate (300 µl per well in triplicates) using SpectraMax-M5 plate reader at 595 nm. $FeCl_3$, iron standards are always included (0-4 µg /ml). Fe standards require only about 30 minutes but can be kept in 1x working solution for at least a week without any significant loss of the signal.

Alternatively for faster processing, the samples could be digested first with concentrated nitric acid:

NANOPARTICLES:

Add 5 µl of nanoparticles to 95 µl of PBS, add 100 µl of concentrated nitric acid (use SealRight® 1.5 ml tubes to avoid any potential leakage), transfer to 70-80º C heat block and heat for 2 hrs. Turn off the heat block, allow to cool, remove the samples and spin at 5000 rpm for 5 min in a microcentrifuge. Neutralize by slowly adding 160 µl of 10 N NaOH. Use 100 µl for ferene-s assay (if the original sample has a high concentration of Fe use less and add PBS to final volume of 100 µl, also see notes) .

CELLS LOADED WITH NANOPARTICLES:

Wash the cells with PBS (make sure to count the cells if need to determine the amount of Fe/cell). Transfer the cells to 1.5 ml SealRight® tubes and spin at 5000 rpm for 5 min in a microcentrifuge. Aspirate carefully without disrupting the pellet (could be frozen at 80º C). Resuspend the cells in PBS. To digest the cells add 100 µl of concentrated nitric acid to100 µl of cell suspension and transfer to 70-80º C heat block and heat for 2 hrs. Turn off the heat block, allow to cool, remove the samples and spin at 5000 rpm for 5 min in a microcentrifuge. Neutralize by slowly adding 160 µl of 10 N NaOH.

FERENE-S ASSAY**:**

Add 100 µl of digested and neutralized sample to 900 µl of 1x working solution, mix well and leave at room temperature for at least 30 min. Transfer 300 µl to each well of a 96-well plate in triplicates and read the absorbance at 595 nm.

Include Fe standards with a range of (0-4 µg) such as 0.1 µg, 0.2 µg, 0.5 µg, 1.0 µg, 2.0 µg and 4.0 µg (total Fe in 1x working solution).

Notes: Samples with high concentration of Fe can be further diluted in 1x working solution. SpectraMax-M5 plate reader from Molecular Devices allows for absorbance readings as high as 3. If the absorbance readings are higher than 3 dilute the samples until the readings are ≤ 3. If the

concentration of Fe in the sample is low, all of the digested and neutralized sample can be added to 1000 µl of 1x working solution.

SUPPLEMENTARY FIGURES

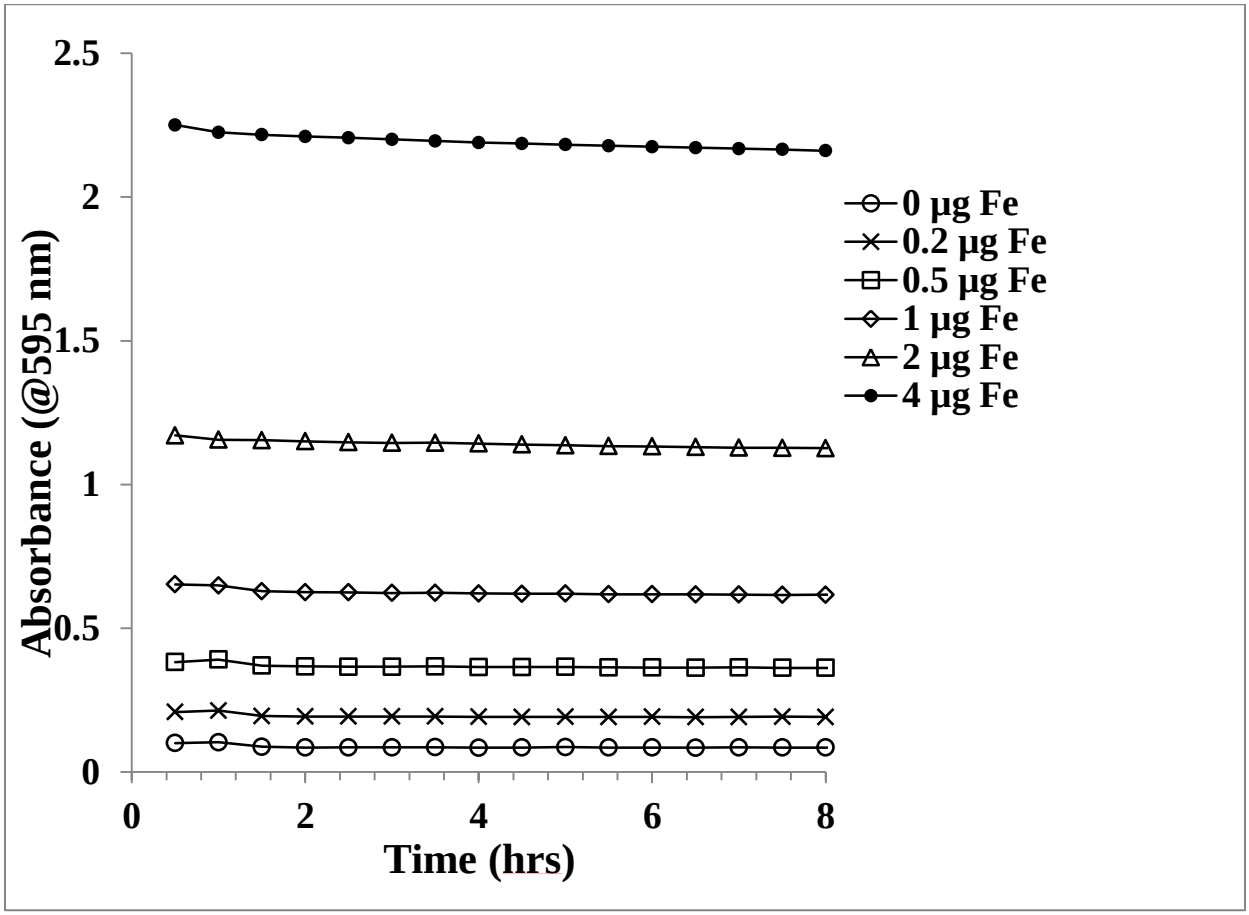

Figure S1: Formation of iron ferene-s complex (Fe^{2+}: ferene-s) in the working solution occurs rapidly when using $FeCl_3$ standards. Tests were conducted at room temperature. Once formed the complex is stable for at least 1 week, see Supplementary Table S6 (below).

SUPPLEMENTARY TABLES

Supplementary Table S1. Molar absorptivity (ε) calculation for ferene-s and ferrozine in the working solution (1 ml cuvette), *A* is the net absorbance obtained from the average of a least 3 experiments. Calculated from Beer-Lambert law $A = \varepsilon cl$, *where* $l = 1cm$.

Iron concentration (c) (μg/ml)	(μM)	*A*	ε ($\mu M^{-1}cm^{-1}$)	($M^{-1}cm^{-1}$)
Ferene-s				
0.0	*0.00*	*0.00*		
0.1	*1.79*	*0.07*	*0.036634*	*36634*
0.2	*3.58*	*0.12*	*0.034547*	*34547*
0.5	*8.95*	*0.31*	*0.035545*	*35545*
1	*17.9*	*0.61*	*0.034462*	*34462*
2	*35.8*	*1.26*	*0.035317*	*35317*
4	*71.6*	*2.48*	*0.034657*	*34657*
			Mean ± SD	*35194 ± 831*
Ferrozine				
0.0	*0.0*	*0.00*		
0.1	*1.79*	*0.05*	*0.028686*	*28686*
0.2	*3.58*	*0.10*	*0.027336*	*27336*
0.5	*8.95*	*0.25*	*0.027520*	*27520*
1	*17.9*	*0.49*	*0.027371*	*27371*
2	*35.8*	*0.98*	*0.027453*	*27453*
4	*71.6*	*1.96*	*0.027390*	*27390*
			Mean ± SD	*27626 ± 523*

Supplementary Table S2. Molar absorptivity calculation for ferene-s and ferrozine in the working solution (96-w plate)), *A* is the net absorbance obtained from the average of a least 3 experiments (calculated from Beer-Lambert law $A = \varepsilon cl$, assuming that l is ~0.88 cm for a volume 300 µl in each well).

Iron concentration (c)		A	ε	
(µg/ml)	(µM)		($\mu M^{-1}cm^{-1}$)	($M^{-1}cm^{-1}$)
Ferene-s				
0.0	*0.00*	*0.00*		
0.1	*1.79*	*0.06*	*0.0354854*	*35485*
0.2	*3.58*	*0.10*	*0.0324236*	*32424*
0.5	*8.95*	*0.27*	*0.0336640*	*33664*
1	*17.9*	*0.52*	*0.0323451*	*32345*
2	*35.8*	*1.04*	*0.0325728*	*32553*
4	*71.6*	*2.04*	*0.0324536*	*32454*
			Mean ± SD	*33157 ± 1242*
Ferrozine				
0.0	*0.0*	*0.00*		
0.1	*1.79*	*0.05*	*0.0292548*	*29255*
0.2	*3.58*	*0.09*	*0.0273937*	*27394*
0.5	*8.95*	*0.29*	*0.0270728*	*27073*
1	*17.9*	*0.41*	*0.0267722*	*26772*
2	*35.8*	*0.54*	*0.0268541*	*26854*
4	*71.6*	*1.60*	*0.0266060*	*26606*
			Mean ± SD	*27326 ± 984*

Supplementary Table S3. Net absorbance readings of iron standards for ferene-s in the working solution (1 ml cuvette). Net absorbance = absorbance of the sample – absorbance of the working solution.

Iron concentration (c)		*Net absorbance*						
(*µ*g/ml)	(*µ*M)	*exp1*	*exp2*	*exp3*	*exp4*	*mean*	*SD*	*% CV*
0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
0.1	*1.79*	*0.069*	*0.063*	*0.065*	*0.065*	*0.066*	*0.0025*	*3.83*
0.2	*3.58*	*0.126*	*0.118*	*0.125*	*0.125*	*0.124*	*0.0037*	*2.96*
0.5	*8.95*	*0.313*	*0.314*	*0.316*	*0.331*	*0.318*	*0.0081*	*2.53*
1.0	*17.9*	*0.616*	*0.582*	*0.620*	*0.651*	*0.617*	*0.0278*	*4.50*
2.0	*35.8*	*1.235*	*1.268*	*1.258*	*1.293*	*1.265*	*0.0262*	*2.07*
4.0	*71.6*	*2.468*	*2.498*	*2.346*	*2.619*	*2.482*	*0.1224*	*4.52*
Slope		*0.616*	*0.626*	*0.590*	*0.645*	*0.622*	*0.0267*	*4.30*

Supplementary Table S4. Absorbance readings of iron standards for ferene-s in the working solution (96-w plate). Net absorbance = absorbance of the sample – absorbance of the working solution, total net absorbance = net absorbance × total volume/volume of sample in each well (1000/300).

		exp1	*exp2*	*exp3*	*exp4*	*mean*	*SD*	*% CV*
Iron concentration (c) (μg/ml)	(μM)			*Net absorbance*				
0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
0.1	*1.79*	*0.062*	*0.053*	*0.053*	*0.058*	*0.057*	*0.0044*	*7.71*
0.2	*3.58*	*0.104*	*0.100*	*0.100*	*0.109*	*0.103*	*0.0043*	*4.13*
0.5	*8.95*	*0.264*	*0.266*	*0.265*	*0.277*	*0.268*	*0.0061*	*2.26*
1.0	*17.9*	*0.515*	*0.484*	*0.515*	*0.546*	*0.515*	*0.0253*	*4.91*
2.0	*35.8*	*1.020*	*1.042*	*1.022*	*1.065*	*1.037*	*0.0210*	*2.02*
4.0	*71.6*	*2.021*	*2.062*	*1.941*	*2.166*	*2.048*	*0.0221*	*4.57*
Slope		*0.504*	*0.516*	*0.487*	*0.540*	*0.511*	*0.0267*	*4.31*
Iron concentration (c) (μg/ml)	(μM)			*Total net absorbance*				
0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
0.1	*1.79*	*0.207*	*0.177*	*0.177*	*0.193*	*0.187*	*0.0145*	*7.78*
0.2	*3.58*	*0.347*	*0.333*	*0.333*	*0.363*	*0.338*	*0.0142*	*4.22*
0.5	*8.95*	*0.880*	*0.887*	*0.883*	*0.923*	*0.883*	*0.0202*	*2.29*
1.0	*17.9*	*1.717*	*1.613*	*1.717*	*1.820*	*1.682*	*0.0844*	*5.02*
2.0	*35.8*	*3.400*	*3.473*	*3.407*	*3.550*	*3.427*	*0.0700*	*2.04*
4.0	*71.6*	*6.734*	*6.873*	*6.470*	*7.220*	*6.693*	*0.3121*	*4.66*
Slope		*1.680*	*1.721*	*1.623*	*1.798*	*1.675*	*0.0737*	*4.40*

Supplementary Table S5. Net absorbance readings of iron standards for ferene-s in the working solution (96-w plate with pathcheck correction). Net absorbance = absorbance of the sample – absorbance of the working solution. A cuvette containing working solution was used as the reference.

Iron concentration (c)		*Net absorbance*					
(*µ*g/ml)	(*µ*M)	*exp1*	*exp2*	*exp3*	*mean*	*SD*	*% CV*
0	*0*	*0*	*0*	*0*	*0*	*0*	*0*
0.1	*1.79*	*0.069*	*0.073*	*0.075*	*0.072*	*0.0031*	*4.22*
0.2	*3.58*	*0.136*	*0.135*	*0.139*	*0.137*	*0.0021*	*1.52*
0.5	*8.95*	*0.317*	*0.330*	*0.321*	*0.322*	*0.0067*	*2.06*
1.0	*17.9*	*0.646*	*0.664*	*0.642*	*0.651*	*0.0117*	*1.80*
2.0	*35.8*	*1.288*	*1.264*	*1.263*	*1.271*	*0.0141*	*1.11*
4.0	*71.6*	*2.466*	*2.493*	*2.496*	*2.485*	*0.0165*	*0.66*
Slope		*0.618*	*0.621*	*0.622*	*0.621*	*0.0023*	*0.36*

Supplementary Table S6. Stability of absorbance readings of iron standards for ferene-s in working solution for up to 21 days (96-w plate).

		day 1	day 6	day 12	day 21	mean	SD	% CV
Iron concentration (c)				*Net absorbance*				
(µg/ml)	(µM)							
0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
0.1	*1.79*	*0.060*	*0.050*	*0.041*	*0.057*	*0.052*	*0.0084*	*16.2*
0.2	*3.58*	*0.105*	*0.093*	*0.086*	*0.095*	*0.095*	*0.0078*	*8.28*
0.5	*8.95*	*0.263*	*0.246*	*0.226*	*0.240*	*0.244*	*0.0153*	*6.29*
1.0	*17.9*	*0.515*	*0.495*	*0.472*	*0.483*	*0.491*	*0.0184*	*3.74*
2.0	*35.8*	*1.021*	*0.987*	*0.964*	*0.981*	*0.988*	*0.0239*	*2.42*
4.0	*71.6*	*2.049*	*2.006*	*2.003*	*2.010*	*2.017*	*0.0215*	*1.07*
Slope		*0.510*	*0.501*	*0.501*	*0.501*	*0.504*	*0.0047*	*0.94*
Iron concentration (c)				*Total net absorbance*				
(µg/ml)	(µM)							
0	*0*	*0*	*0*	*0*	*0*	*0*	*0*	*0*
0.1	*1.79*	*0.200*	*0.167*	*0.137*	*0.190*	*0.173*	*0.0282*	*16.24*
0.2	*3.50*	*0.350*	*0.310*	*0.287*	*0.317*	*0.316*	*0.0262*	*8.28*
0.5	*8.95*	*0.877*	*0.820*	*0.753*	*0.800*	*0.813*	*0.0511*	*6.29*
1.0	*17.9*	*1.717*	*1.650*	*1.573*	*1.610*	*1.638*	*0.0614*	*3.74*
2.0	*35.8*	*3.403*	*3.290*	*3.213*	*3.270*	*3.294*	*0.0797*	*2.42*
4.0	*71.6*	*6.830*	*6.687*	*6.677*	*6.700*	*6.723*	*0.0718*	*1.07*
Slope		*1.702*	*1.671*	*1.671*	*1.671*	*1.681*	*0.0157*	*0.94*

Supplementary Table S7. *Total net absorbance* of iron standards and the slope of standard curves in working solution (96-w plate).

Fe (μg/ml)	*exp1*	*exp2*	*exp3*	*exp4*	*exp5*	*mean*	*SD*
0	*0*	*0*	*0*	*0*	*0*	*0*	*0*
0.1	*0.176*	*0.213*	*0.190*	*0.190*	*0.264*	*0.207*	*0.035*
0.2	*0.311*	*0.377*	*0.364*	*0.324*	*0.458*	*0.367*	*0.057*
0.5	*0.946*	*0.931*	*0.824*	*0.868*	*0.995*	*0.913*	*0.067*
1.0	*1.782*	*1.794*	*1.623*	*1.730*	*1.848*	*1.756*	*0.085*
2.0	*3.487*	*3.516*	*3.329*	*3.468*	*3.454*	*3.451*	*0.072*
4.0	*6.736*	*7.277*	*6.639*	*6.867*	*6.860*	*6.867*	*0.243*
Slope	*1.688*	*1.806*	*1.657*	*1.712*	*1.693*	*1.712*	*0.057*