Silvered Conical-Bottom 96-well Plates: Enhanced Low Volume Detection and the Metal-Enhanced Fluorescence Volume/Ratio Effect

Rachael Knoblauch and Chris D. Geddes*

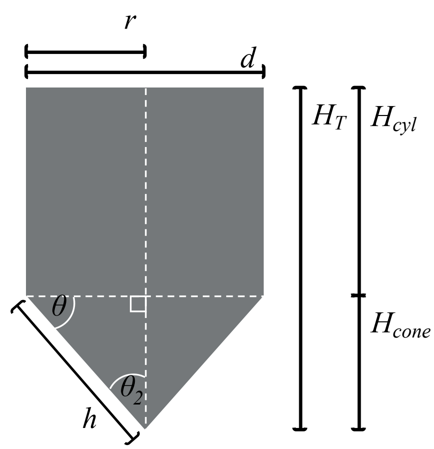
Institute of Fluorescence and the Department of Chemistry and Biochemistry, University of Maryland Baltimore County, 701 E Pratt Street, Baltimore, Maryland, 21202, USA

*All correspondence: geddes@umbc.edu

Supplemental Material

Extraction of Measured MEF and Resulting Corrected MEF Values

S2.3.1. Determining well dimensions. To determine the well dimensions for subsequent calculations, information for the well plates (Nunc™, conical) was first accessed and a metric volume of 450 µL was reported for the wells. The wells were then measured as shown in Scheme S1 below, with the following reported values: $d = 0.8$ cm, $H_T = 1.3$ cm, $H_{cyl} = 0.7$ cm, $h = 0.55$ cm. H_{cone} was calculated from H_{cyl} and H_T, and is reported to be 0.6 cm.



Scheme S1. Diagram of the conical wells depicting dimensions and corresponding variable labels.

These measured values were then confirmed using well volumes of 450 µL, 80 µL, and 370 µL for the total well (V_T), cone portion (V_{cone}), and cylindrical portion (V_{cyl}) respectively, using equations S1-S5. In these equations, r is the radius of the sample well and is given as 0.4 cm; this is a measured value, which is assumed to be true as the well diameter measurement (d) was an easily obtained quantity with no recorded variation between wells.

$$V_{cyl} = \pi r^2 H_{cyl} \quad \text{(Equation S1)}$$

$$V_{cone} = \pi r^2 \frac{H_{cone}}{3} \quad \text{(Equation S2)}$$

$$\theta = \tan^{-1}\left(\frac{H_{cone}}{r}\right) \quad \text{(Equation S3)}$$

$$\theta_2 = \tan^{-1}\left(\frac{r}{H_{cone}}\right) \quad \text{(Equation S4)}$$

$$h = \frac{H_{cone}}{\sin\theta} \qquad \text{(Equation S5)}$$

The variables are defined in Table S1 below, with values included for both the measured and from volume calculations.

Table S1. Conical Well Dimensions from Two Measurement Strategies.

Variable	**Measured**	**From Volume**	**Average**
d (well width, diameter)	0.80 cm	N/A	0.80 cm
r (1/2 well width, radius)	0.40 cm	N/A	0.40 cm
H_T (height of total well)	1.15 cm	1.21_3 cm	1.18_1 cm
H_{cyl} (height of cylindrical portion)	0.70 cm	0.73_6 cm	0.71_8 cm
H_{cone} (height of cone portion)	0.45 cm	0.47_7 cm	0.46_4 cm
h (hypotenuse of cone)	0.55 cm	0.63_8 cm	0.59_4 cm
θ	N/A	$48._4°$	$48._4°$
θ_2	N/A	$41._6°$	$41._6°$

Given the variation between values, the magnitudes for each variable were averaged and used as constants in subsequent calculations.

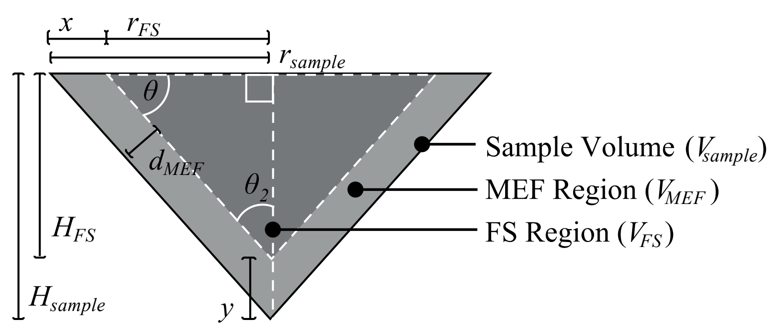
S2.3.2. Correcting the Metal Enhancement Factor. Values defined as "MEF" in this publication are reported as described in Section 2.3 of the main text and were calculated considering the entire sample solution; however, only fluorophores within an ~50 nm range of the plasmonic substrate will couple. As such, we have generated corrected MEF values (MEF_C) for each data point collected that consider only that portion of the solution which is in this MEF region. In addition, we have calculated MEF_C values per mole of fluorophore, indicated as MEF_C mol^{-1}. The calculations for these quantities are described below.

In section S2.3.1. we describe a method for determining the dimensional properties of the cone portion of the conical well plates; however, in order to evaluate the MEF region volume (V_{MEF}), these properties must be used to ascertain the height (H_{sample}) and radius (r_{sample}) as the overall sample volume is decreased. Using Equations S6 and S7 and entering relevant sample volumes for V_{sample} (1-5, 20, 50, 80 μL), these values can be calculated.

$$r_{sample} = \sqrt[3]{\frac{3 * V_{sample}}{\pi \tan\theta}} \qquad \text{(Equation S6)}$$

$$H_{sample} = r_{sample} \tan(\theta) \quad \text{(Equation S7)}$$

The volume of free-space, or un-coupled, fluorophore solution (V_{FS}) can then be calculated using these values and the additional variables shown in Scheme S2.



Scheme S2. Diagram of sample in conical wells depicting dimensions and variables. Diagram is not drawn to scale.

Using these variables and setting d_{MEF} equal to 50 nm, the MEF volume can be determined by Equations S8-12.

$$x = \frac{d_{MEF}}{\sin\theta} \quad \text{(Equation S7)}$$

$$y = \frac{d_{MEF}}{\sin\theta_2} \quad \text{(Equation S8)}$$

$$r_{FS} = r_{sample} - x \quad \text{(Equation S9)}$$

$$H_{FS} = H_{sample} - y \quad \text{(Equation S10)}$$

$$V_{FS} = \pi {r_{FS}}^2 (\frac{H_{FS}}{3}) \quad \text{(Equation S11)}$$

$$V_{MEF} = V_{sample} - V_{FS} \quad \text{(Equation S12)}$$

Additionally, the percentage of solution that may couple to silver for plasmonic enhancement (*%MEF*) can be determined from the volumes provided and calculated according to Equation S13.

$$\%MEF = \frac{V_{MEF}}{V_{sample}} * 100 \quad \text{(Equation S13)}$$

These %MEF values are summarized in Table S2.

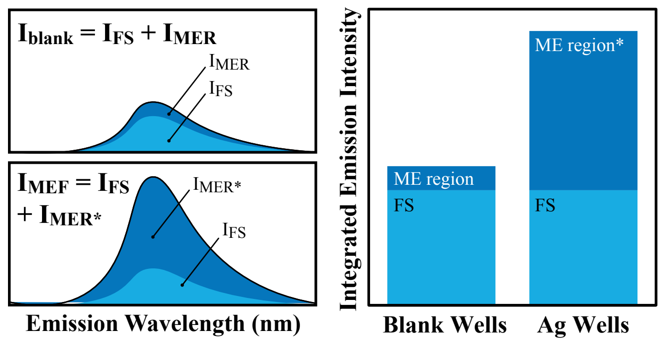
Table S2. Percent of Fluorophore Solutions in MEF Region.

Sample Volume (µL)	%MEF
1	0.021%
2	0.017%
3	0.015%
4	0.013%
5	0.012%
20	0.008%
50	0.006%
80	0.005%

To obtain the corrected factors (MEF_C), first the MEF factors were extracted from Figures 4 and 6. The individual graphs were magnified and measured for scale. The "y" height (in cm) of each visible point was then determined against the x-axis and converted to a corresponding MEF value depending on the scale ratio for each plot. These values were calculated originally as described in Section 2.3 of the main text, according to Equation S14, where I_{MEF} and I_{blank} are the peak intensities from fluorophore in the silvered and blank conical wells respectively.

$$MEF = \frac{I_{MEF}}{I_{blank}} \quad \text{(Equation S14)}$$

However, the enhancement observed, in truth, is only due to that volume of fluorophore within the MEF region and can be visualized by considering Scheme S3, where the relative contributions to fluorescence intensity are shown for both the blank and silvered wells, assuming that intensity can be considered additive between well volume regions. In the case of fluorescence intensity from the blank (I_{blank}), the enhancement region intensity (I_{MER}) contributes little to overall emission intensity compared to the free-space region intensity (I_{FS}). In the silvered wells, however, an enhanced intensity (I_{MEF}) is observed. This is a consequence of the plasmonic amplification of fluorophore *only* within the enhancement region for silvered wells (I_{MER^*}); however, the intensity from the free-space intensity remains constant between the blank and silvered wells, as this volume is unaffected by the plasmonic material.



Scheme S3. Diagram depicting enhancement of total fluorescence and relative intensity contribution from enhancement region and free-space volumes. Spectra and bar graphs are intended to be representative and are not drawn to scale.

This phenomenon is corrected for mathematically by Equation S15 through S17.

$$I_{MER} = I_{blank} - I_{FS} \quad \text{(Equation S15)}$$

$$I_{MER*} = I_{MEF} - I_{FS} \quad \text{(Equation S16)}$$

$$MEF_C = \frac{I_{MER*}}{I_{MER}} \quad \text{(Equation S17)}$$

The corrected MEF factor (MEF_C), therefore, is only due to the fluorophores contained in the MEF region. To obtain these values, Equation S17 is modified to Equation S17.1 using the relationships described by Equations 18 and 19.

$$MEF_C = (\frac{100}{\%MEF})(MEF - 1) \quad \text{(Equation S17.1)}$$

$$\frac{V_{MEF}}{V_{sample}} = \frac{\%MEF}{100} = \frac{I_{MER}}{I_{blank}} \quad \text{(Equation S18)}$$

$$MEF = \frac{I_{MEF}}{I_{blank}} \quad \text{(Equation S19)}$$

For these equations, the intensity from free space fluorophores (I_{FS}) was assumed to be approximately equal to the peak intensities from the blank wells (I_{blank}), as the MEF regions only compose <0.1% according to Table S2. These values were then determined per mole of fluorophore ($MEF_C\ mol^{-1}$) using the calculated MEF region volumes for each sample volume and the concentration of fluorophore used.

Figure S1. Photographs of Rhodamine 6G solutions in conical versus flat bottom 96-well plates and the resulting detectability limits in the plate reader.

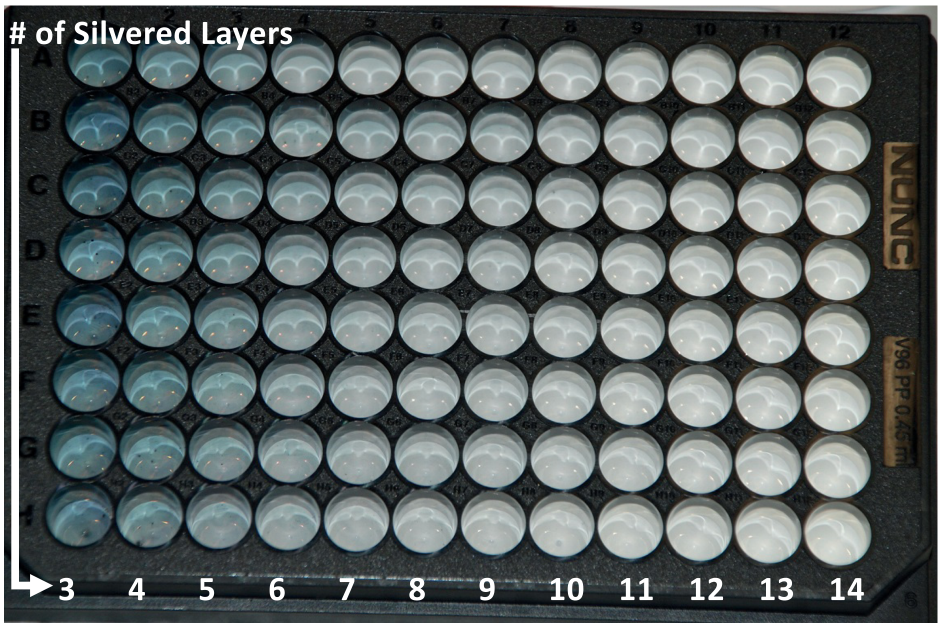
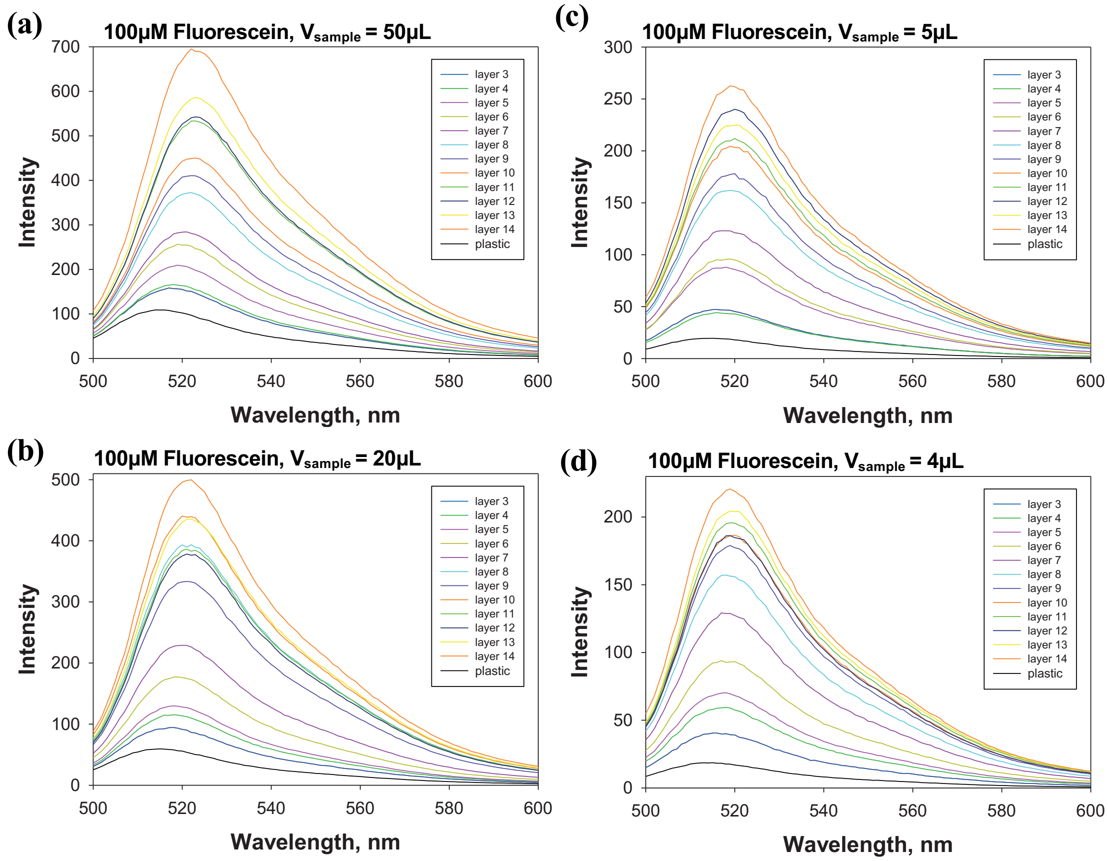
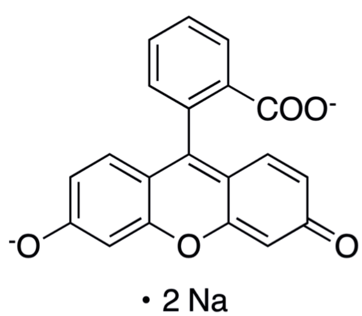


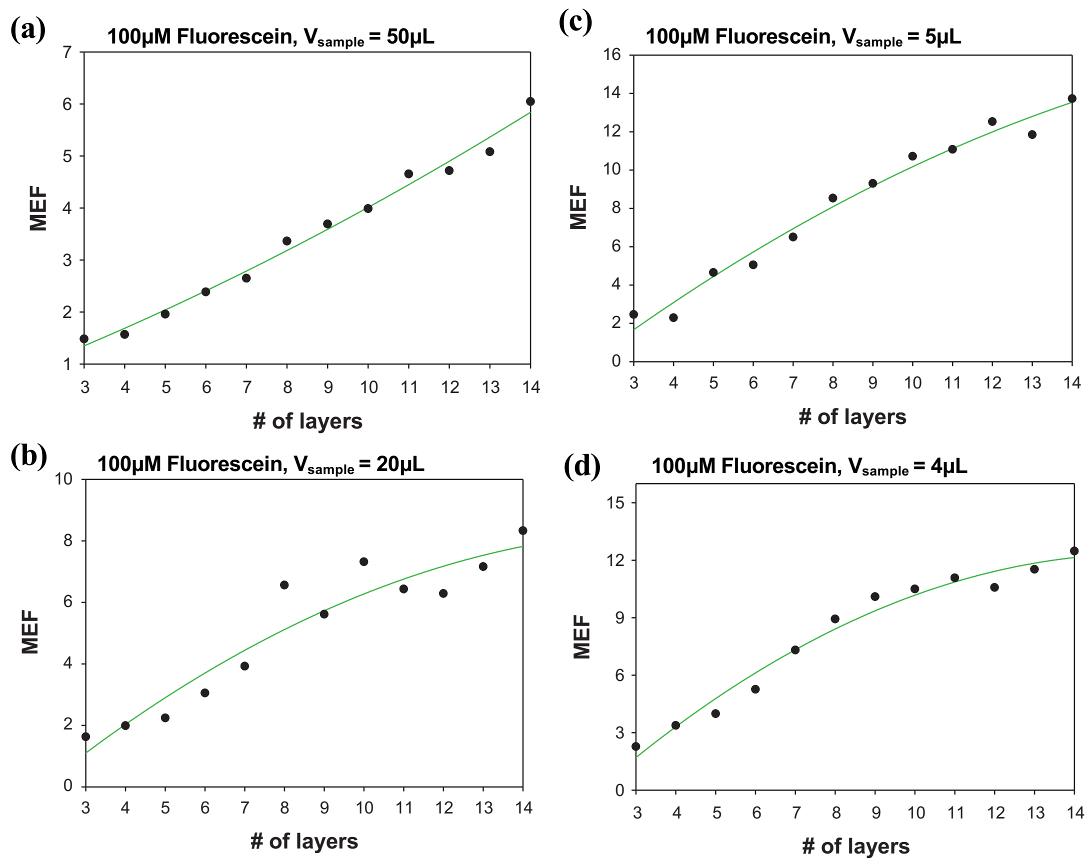
Figure S2. Photograph of conical bottom well plate with various layers of silver deposition.

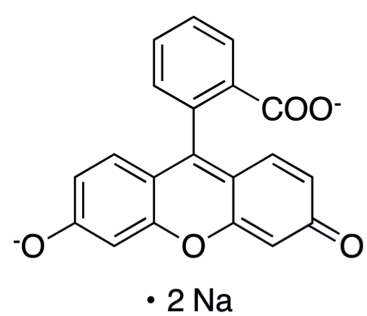




Fluorescein

Figure S3. Comparison of metal-enhanced fluorescence (MEF) from emission spectra (λ_{ex} = 490 nm) for 100µM fluorescein in conical bottom wells with various silvered layers. Samples are analyzed for *A)* 50µL, *B)* 20µL, *C)* 5µL, and *D)* 4µL volumes.





Fluorescein

Figure S4. Comparison of metal-enhancement factors (MEF) from emission spectra (λ_{ex} = 490 nm, λ_{em} = 518 nm) for 100µM Fluorescein in conical bottom wells with various silvered layers. Samples are analyzed for *A)* 50µL, *B)* 20µL, *C)* 5µL, and *D)* 4µL volumes.

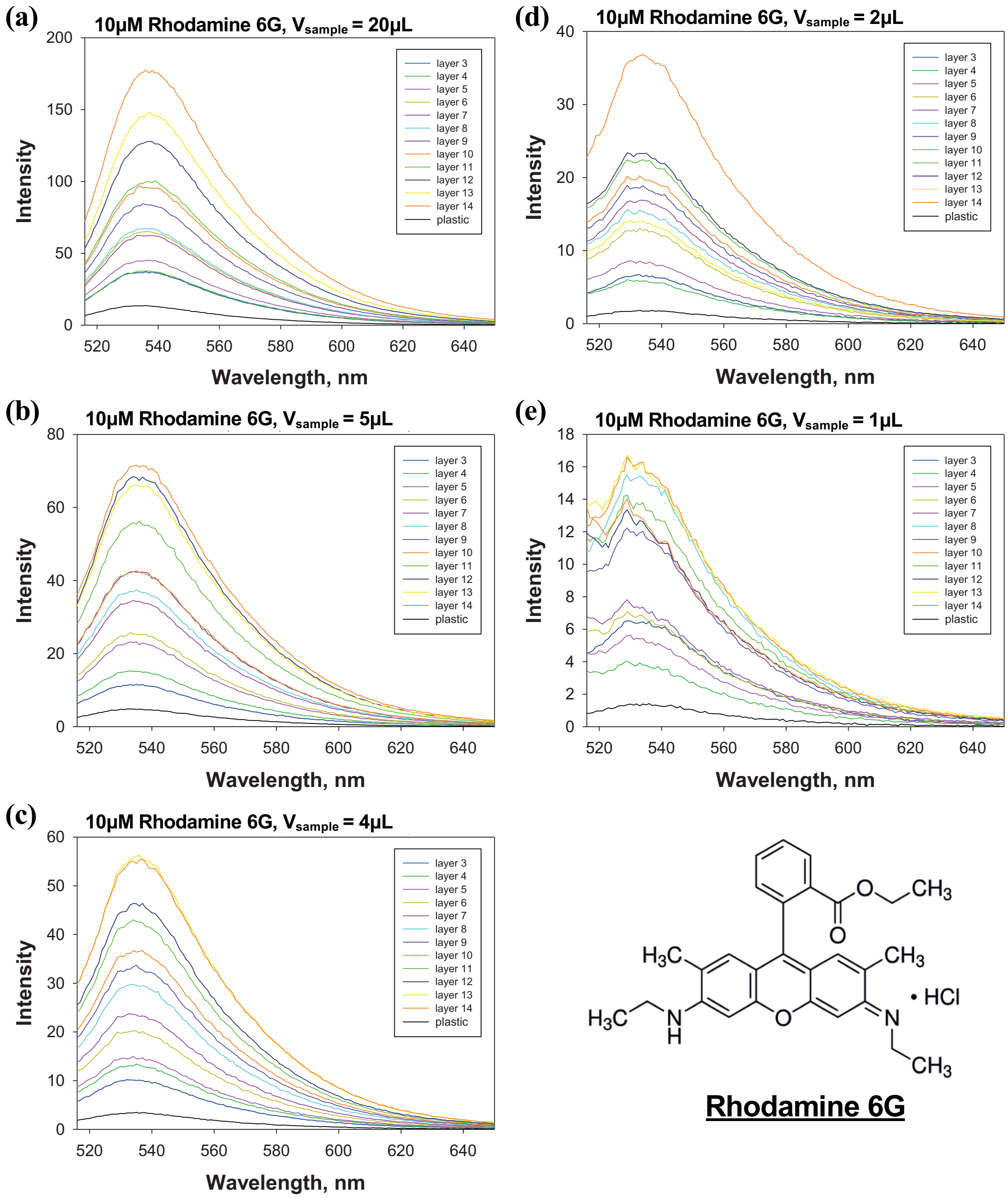


Figure S5. Comparison of metal-enhanced fluorescence (MEF) from emission spectra (λ_{ex} = 506 nm) for 10μM Rhodamine 6G in conical bottom wells with various silvered layers. Samples are analyzed for *A)* 20μL, *B)* 5μL, *C)* 4μL, *D)* 2μL, and *E)* 1μL volumes.

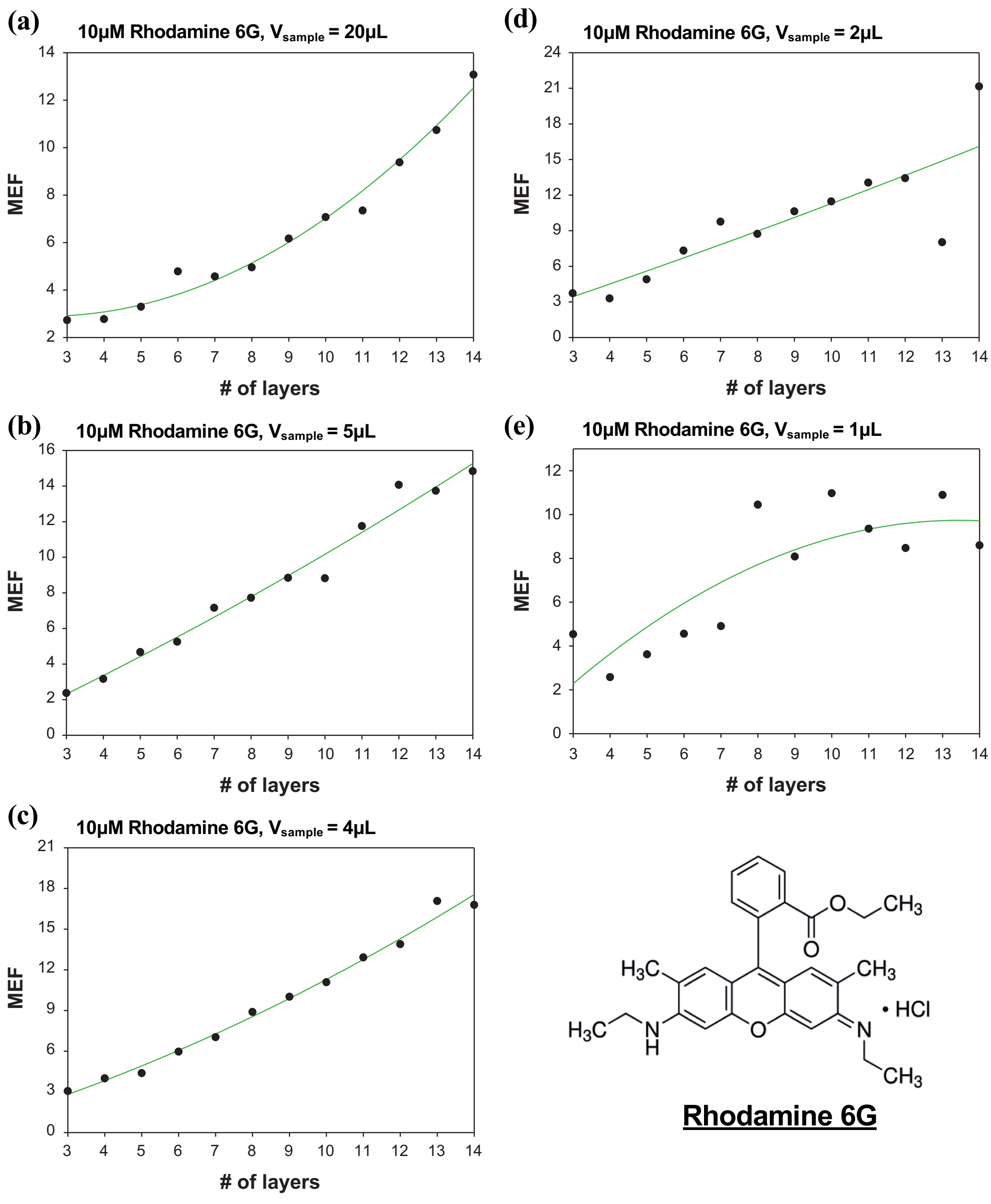


Figure S6. Comparison of metal-enhancement factors (MEF) from emission spectra (λ_{ex} = 506 nm, λ_{em} = 535.97 nm) for 10μM Rhodamine 6G in conical bottom wells with various silvered layers. Samples are analyzed for *A)* 20μL, *B)* 5μL, *C)* 4μL, *D)* 2μL, and *E)* 1μL volumes.

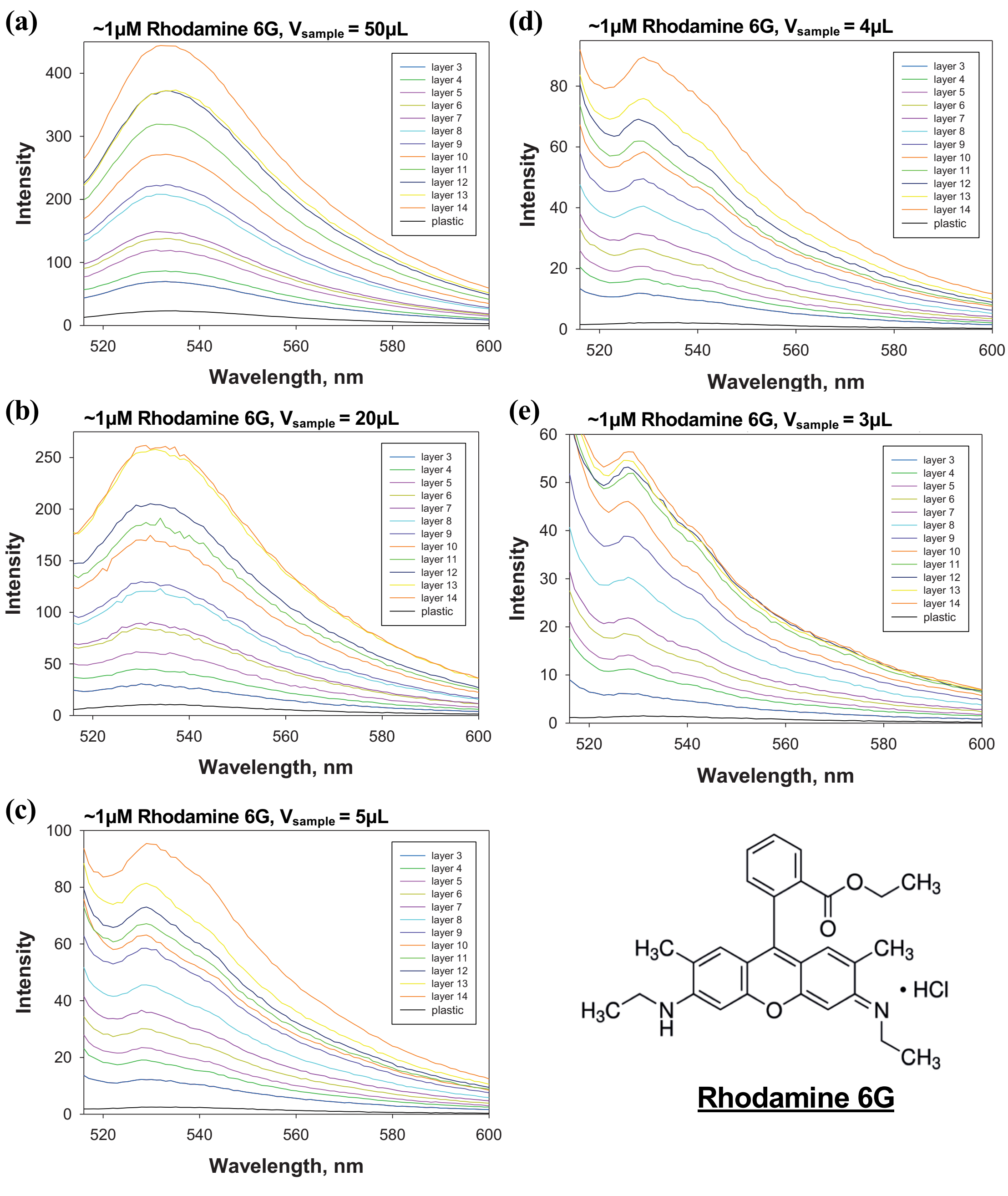


Figure S7. Comparison of metal-enhanced fluorescence (MEF) from emission spectra (λ_{ex} = 506 nm) for ~1µM Rhodamine 6G in conical bottom wells with various silvered layers. Samples are analyzed for *A)* 50µL, *B)* 20µL, *C)* 5µL, *D)* 4µL, and *E)* 3µL volumes.

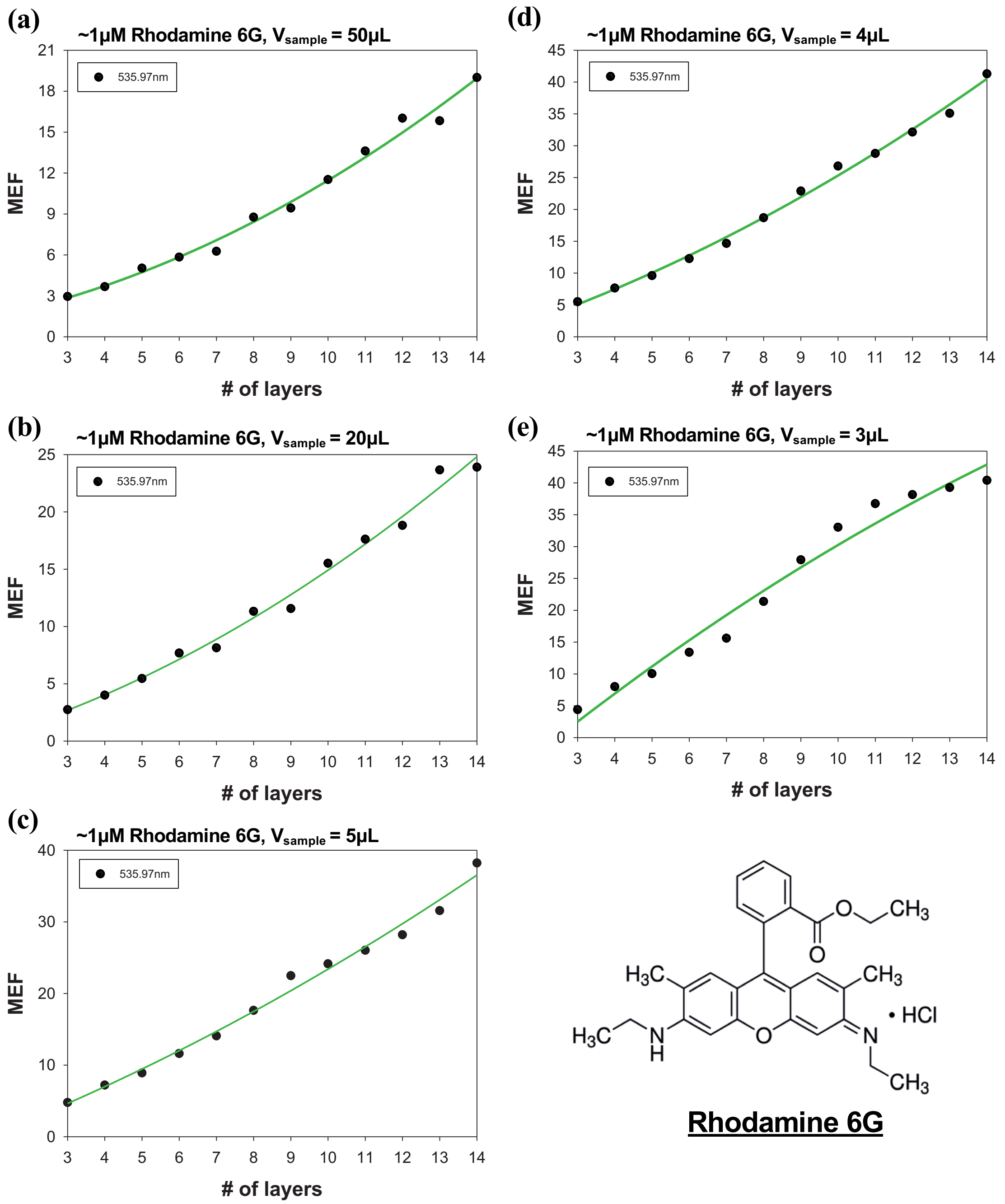


Figure S8. Comparison of metal-enhancement factors (MEF) from emission spectra (λ_{ex} = 506 nm, λ_{em} = 535.97 nm) for ~1μM Rhodamine 6G in conical bottom wells with various silvered layers. Samples are analyzed for *A)* 50μL, *B)* 20μL, *C)* 5μL, *D)* 4μL, and *E)* 3μL volumes.

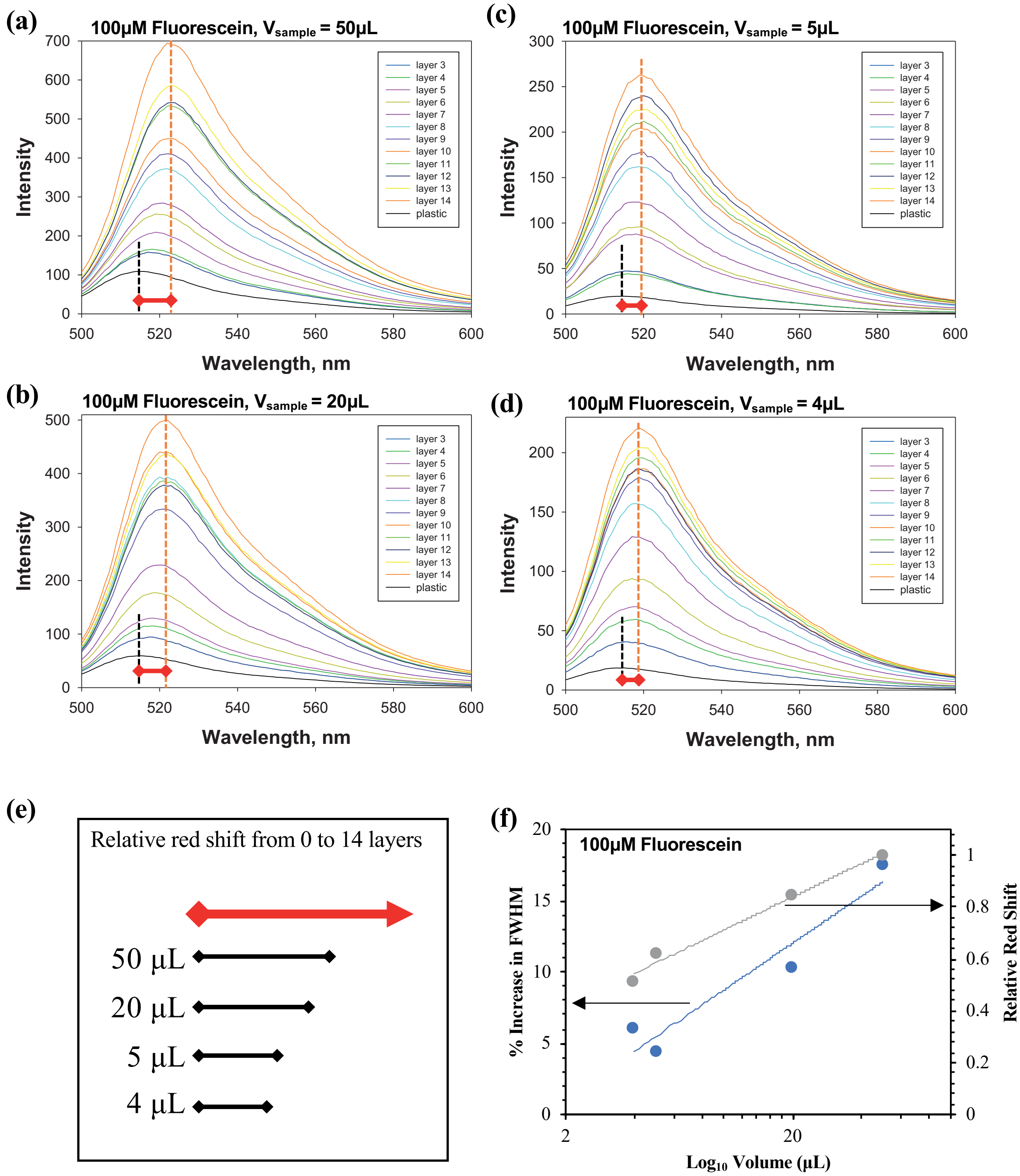


Figure S9. Spectra from Figure S3 with labeled red shift in peak maximum from 0 to 14 layers. Samples are analyzed for *A)* 50μL, *B)* 20μL, *C)* 5μL, and *D)* 4μL volumes. *E)* Overall red shift for each volume summarized. *F)* Log_{10} plot of sample volume against percent increase of full width half maximum (FWHM) from 3 to 14 layers and relative red shift (0 vs 14 layers).

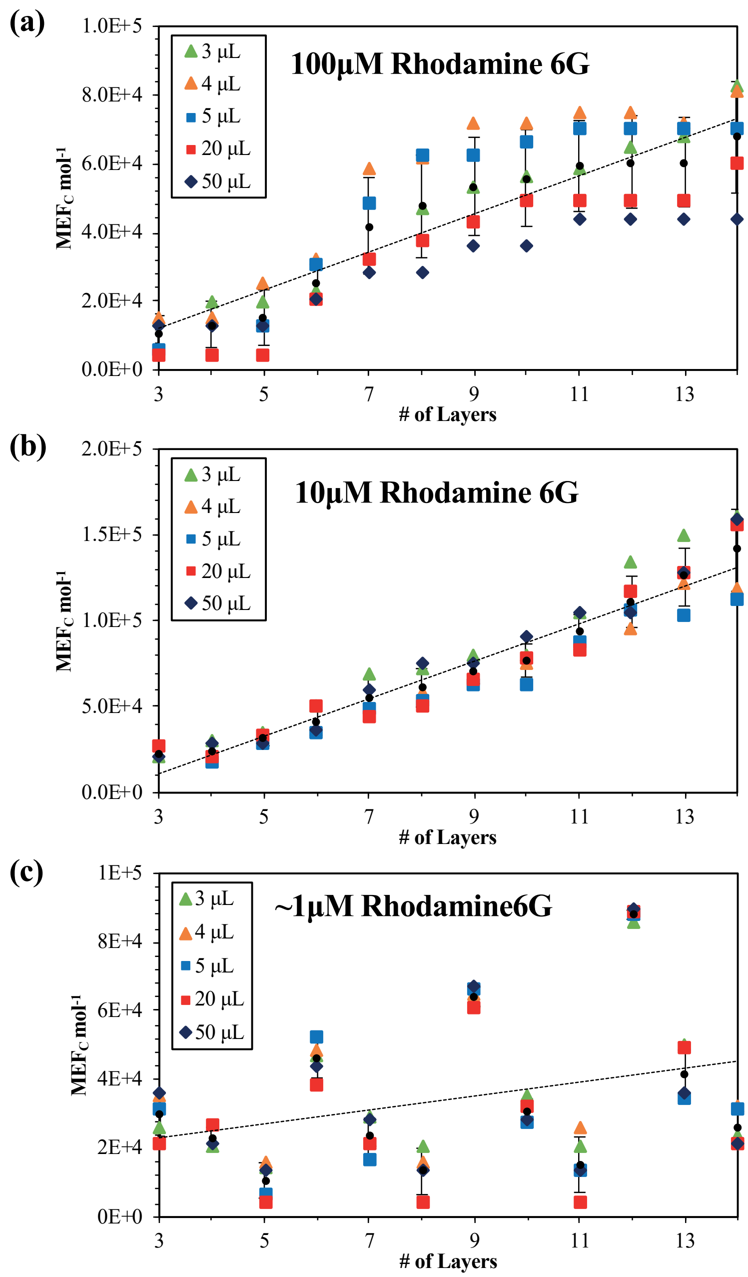


Figure S10. Corrected metal-enhanced fluorescence responses of fluorescein in conical wells with varying silvered layers for fluorescein concentrations of *A)* 100µM, *B)* 10µM and *C)* ~1µM. Corrected metal-enhancement factors (MEF_C) were calculated from corresponding MEF values. Trendlines are from series averages (black circles).

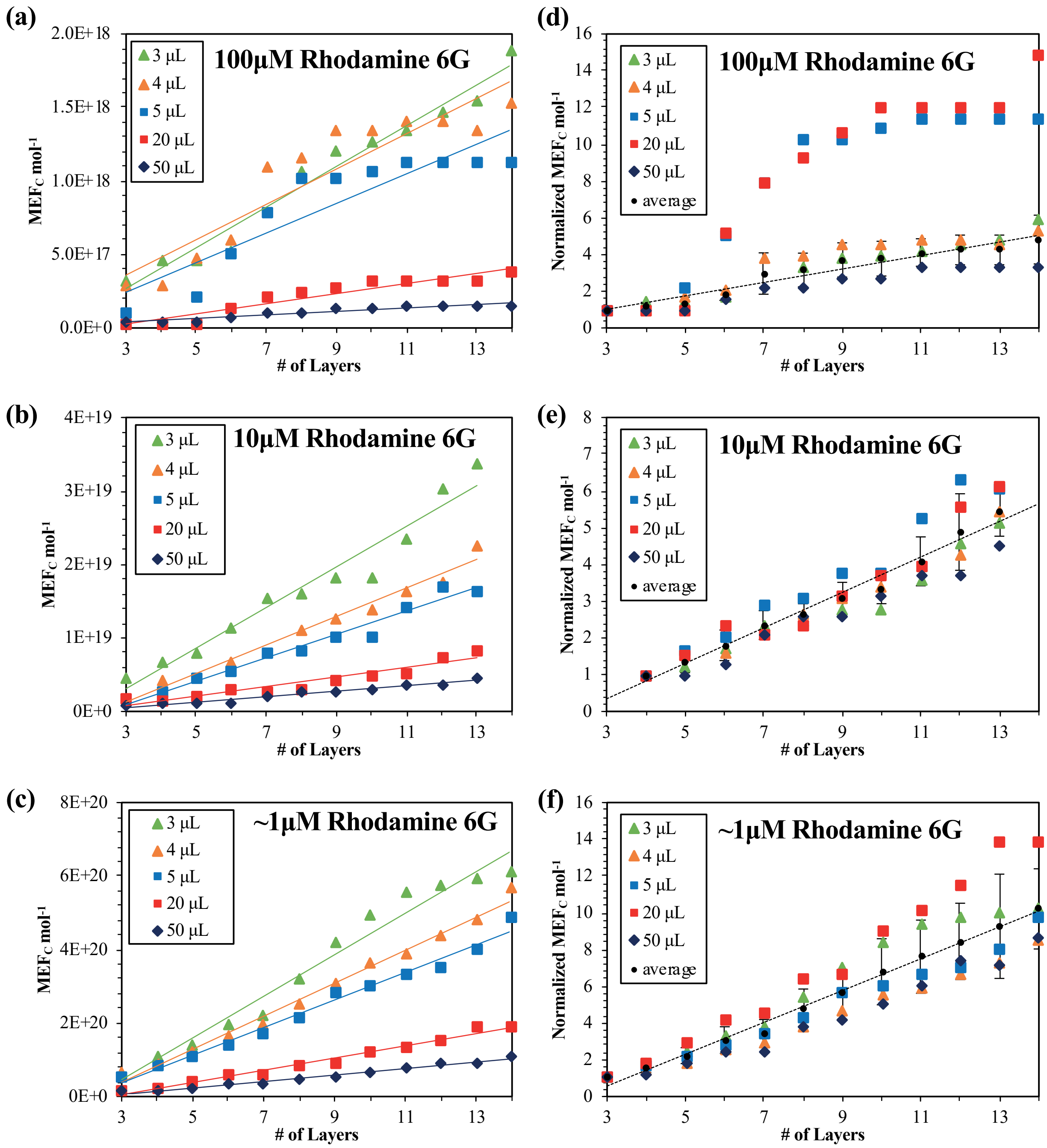


Figure S11. Corrected metal-enhanced fluorescence responses (calculated per mole, $MEF_C\,mol^{-1}$) of fluorescein in conical wells with varying silvered layers for concentrations of *A)* 100µM, *B)* 10 µM and *C)* ~1µM. Normalized plots are included for concentrations of *D)* 100µM, *E)* 10 µM and *F)* ~1µM. Trendlines are from series averages (black circles).