

TruCell

Cell Density Measurement Primer

This technical note explains the physics of measuring the optical loss of a sample in order to deduce its cell density. Optical loss combines both absorbance and scattering effects, both of which are described in de-

tail, and can be modeled as a function of cell concentration using one simple equation. Examples are drawn from practical cell density measurements in real-life industrial applications.

Optical Loss

The TruCell sensor measures the reduction in transmission of the light (called “optical loss”) as it passes across the probe’s measurement gap. This gap has an optical path length (OPL) where the light passes directly

through the fermentation broth. As the optical loss increases, the amount of the transmitted light decreases (see Figure 1). The standard measurement unit of optical loss, L_{opt} , is the absorbance unit (AU).

L_{opt} depends on the wavelength, λ , of the light, and is given by:

$$L_{opt}(\lambda) = A(\lambda) + S(\lambda) + L_{other}(\lambda) = -\log_{10}(I_T(\lambda)/I_0(\lambda)) \text{ [AU]}$$

where: $I_T(\lambda)$ = Light transmitted through sample at wavelength λ

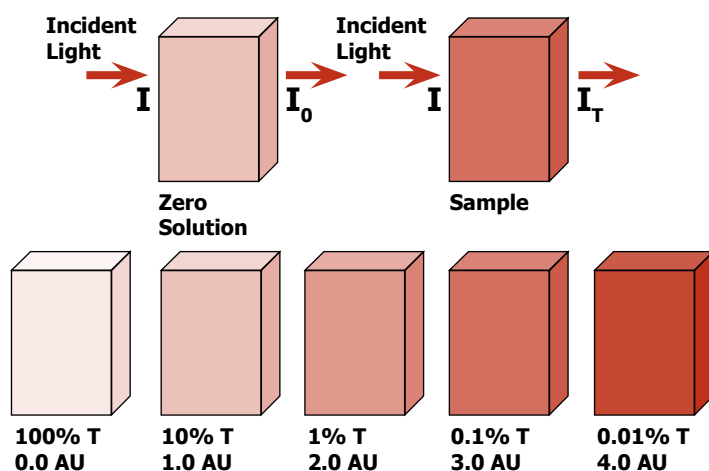
$I_0(\lambda)$ = Light transmitted through zero/reference solution at wavelength λ

$A(\lambda)$ = Optical loss through absorption, also called absorbance, at wavelength λ

$S(\lambda)$ = Optical loss through scattering at wavelength λ , and

$L_{other}(\lambda)$ = Optical loss through non-linear effects or measurement processes at wavelength λ .

Figure 1
Optical absorption principles: (top Figure) reference and sample measurement, and (bottom Figure) samples having increasing absorbance values.



Absorbance

Absorbance, $A(\lambda)$, is a measure of the conversion of radiant energy to heat and chemical energy. It is numerically equal to the fraction of energy absorbed from a light beam over an OPL traveled in an absorbing medium. The Beer-Lambert law defines a linear relationship between $A(\lambda)$ and the OPL, through the molar extinction coefficient, $\mu_\alpha(\lambda, C)$, and absorption coefficient, $\alpha(\lambda, C)$:

$$A(\lambda) = \mu_\alpha(\lambda, C) * OPL = \alpha(\lambda, C) / \ln(10) * OPL$$

The molar extinction and absorption coefficients have units of cm^{-1} , and are proportional to the concentration of the absorbing sample, C , through the extinction coefficient, $\epsilon(\lambda)$, namely, $\alpha(\lambda, C) = \epsilon(\lambda) * C$. Therefore, for a fixed concentration,

absorbance and OPL are proportional, and slope of the line is simply the sample’s molar extinction coefficient, μ_α . The absorption coefficient can also be related to the number density of absorber molecules, N , through the absorption cross section, σ_{abs} , namely, $\alpha(\lambda, N) = \sigma_{abs}(\lambda) * N$.

Figure 2 shows that the absorption coefficient of pure, particle-free water ranges from 0.03 - 0.06 cm^{-1} in the 760-1000 nm wavelength band. At 850 nm, the operating wavelength of the TruCell sensor, the absorption coefficient is 0.03 cm^{-1} , which means that for a 1 cm OPL, $A = 0.03$ AU. Because distilled, deionized water is used as the zero reference liquid, the absorbance of water is negated in TruCell measurements.

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In the case of a purely absorbing sample, absorbance is obtained by measuring the attenuation of the light as it passes through the sample. Namely, absorbance is governed by the following equation:

$$A = \text{Absorbance} = -\log_{10}(I_T/I_0) \text{ [AU]} = -\log_{10}(T) \text{ [AU]}$$

where: I_T = Light transmitted through sample, and

I_0 = Light transmitted through zero/reference solution

T = Light transmittance expressed as decimal percent

Absorbance is simply calculated from the measurement of light transmitted through the sample and referenced to a “zero” solution. For increasing concentrations of the absorbing medium, the amount of the transmitted light decreases, and the absorbance

value increases. Modern spectrometers usually display measured data as either transmittance, %-transmittance, or absorbance. Spectrometer cuvettes used for absorption measurements often have an OPL of 1 cm, so that the absorption coefficient and absorbance readings can be directly compared.

An unknown concentration, C , of a purely absorbing analyte can be determined by measuring the amount of light that a sample absorbs and applying the Beer-Lambert law:

$$C \text{ [M]} = A \text{ [AU]} / \epsilon \text{ [M}^{-1}\text{cm}^{-1}] / \text{OPL [cm]}$$

If the absorption coefficient for a given analyte is not known, the unknown concentration can be determined using a working curve of absorbance versus concentration derived from reference standards. These working curves are obtained by measuring the signal from a series of standards of known concentration. The working curves are then used to determine the concentration of an unknown sample or to calibrate the linearity of the analytical instrument. An example of a working curve for aqueous copper sulfate solution of varying concentration is shown in Figure 3.

Note that the Beer-Lambert law holds only if the analyte being measured has a well-defined absorption feature, and the spectral bandwidth of the light is narrow compared to the linewidth of the sample's absorption feature. Furthermore, Beer-Lambert assumes that the source radiation used by the measurement instrument is both monochromatic and collimated. Finally, there is also the assumption that the sample medium is homogeneous, isotropic, and is free of multiple scattering events. Strictly speaking, for Beer-Lambert to hold - the light measured (e.g.: hitting the detector) must be only the light that has not been scattered. In many cases, especially those involving biological samples, scattering losses dominate over absorbance, leading to a non-linear relationship between concentration and the measured optical loss.

In general, common sources of non-linearity in the relationship between optical loss and concentration include (i.e., where Beer's Law cannot be directly applied):

Figure 2
Absorption coefficient of pure water as a function of wavelength.

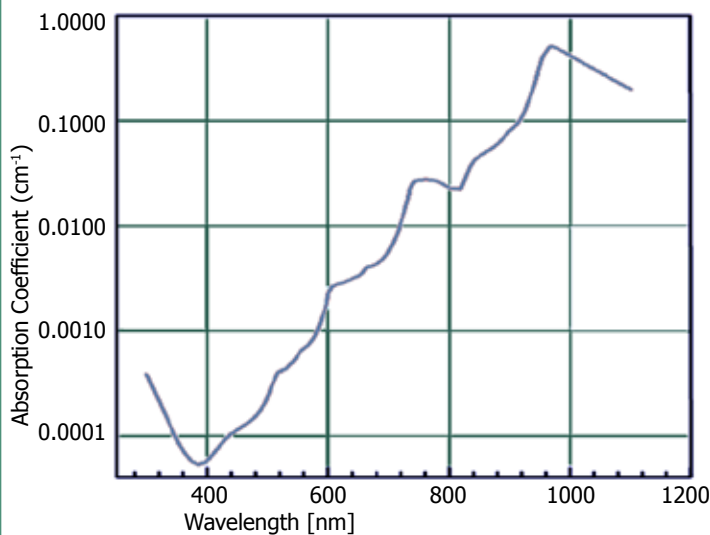
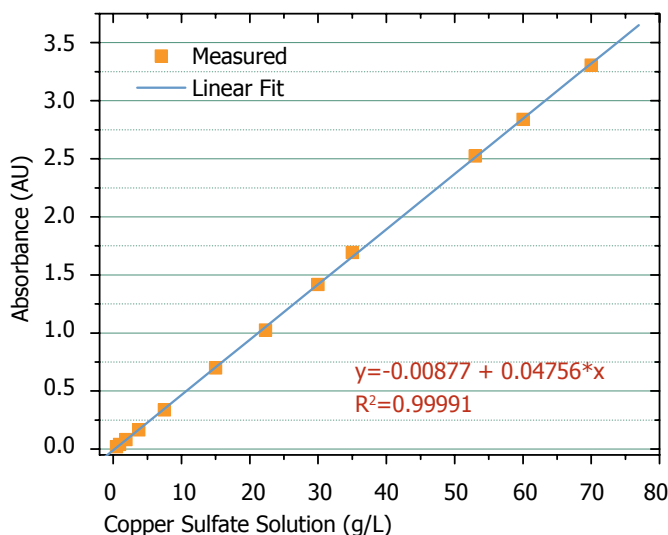
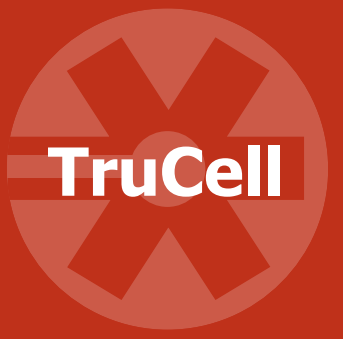


Figure 3
Calibration curve of concentration versus absorbance for copper sulfate solution





- multiple scattering events due to high density of particulates in the sample,
- changes in refractive index and effective OPL at high concentrations,
- fluorescence or phosphorescence of the sample,
- deviations in absorption coefficients at high concentrations (owing to electrostatic interactions between molecules in close proximity),
- shifts in chemical equilibrium as a function of concentration¹,
- stray light entering the measurement system, and
- non-monochromatic radiation (deviations can be minimized by using a relatively flat part of the absorption spectrum such as the maximum of an absorption band)

Scattering

For biological samples, especially those related to fermentation or cell culture, there is a very high degree of non-linearity owing to scattering processes. Scattering is a process by which small particles (such as cells, bacteria, or bubbles) suspended in a medium having a different index of refraction diffuse a portion of the incident radiation in all directions. In other words, scattering changes the direction of light transport, without changing its wavelength, by “dispersing” the photons as they penetrate a turbid sample. The change in spatial distribution of the radiation is converted into a change in detected intensity by a photodiode which has both a fixed active area and location.

The scattering loss is a highly non-linear function of particle concentration, C , sample path length, OPL, particle diameter, d , wavelength, λ , and detector collection (cone) angle, θ , namely, $S = f(C, OPL, d, \lambda, \theta)$.

The scattering loss varies as a function of the ratio of the particle diameter, d , to the wavelength of the radiation, λ . When this ratio is:

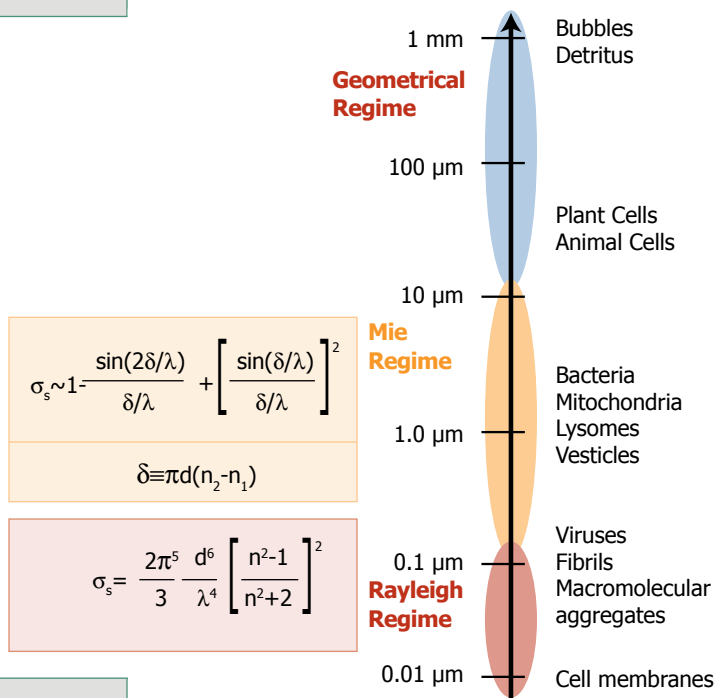
- $(d/\lambda) < 0.1$, the mechanism is Rayleigh scattering, in which the scattering coefficient varies inversely as the fourth power of the wavelength,
- $0.1 < (d/\lambda) < 10$, the scattering varies in a complex fashion described by the Mie theory,
- $(d/\lambda) > 10$, the more simple laws of geometric optics can be applied.

Figure 4 shows the relative sizes of most biological particles.

From Figure 4, we see that most suspended bioparticles (cells, bacteria, and bubbles) in bioreactor media are somewhat larger than the TruCell operating wavelength of $\lambda = 850 \text{ nm}$, so that Mie theory must be applied. Typically, bioparticles scatter about half the incident light energy into a 10-degree forward-directed cone (the active collection area of the TruCell detector), and less than 2.5 percent of it in the backward direction. The TruCell sensor can collect scattered light within a 20-degree forward-directed cone, which maximizes detection of the transmitted light intensity. This large collection angle allows the sensor to achieve high signal to noise ratios even in highly turbid media.

At a fixed wavelength and collection angle, the relationship between scattering loss and particle concentration (or scattering loss and sample path length) generally becomes non-linear when the scattering loss significantly exceeds 1.0 AU. In general, this deviation from linearity occurs when multiple scattering events become prevalent. Furthermore, this relationship depends on the size and optical properties of the particles. For more details on this subject,

Figure 4
Typical sizes of biological particles and the scattering mechanism they would produce for a sensor having a wavelength of 850 nm.





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please refer to the technical note entitled “Scattering Processes – Theory and Experiment.”

At a fixed collection angle, for scattering losses below 1.0 AU, the scattering loss can usually be linearly related to a scattering coefficient, μ_o , and OPL by:

$$S(\lambda) = \mu_o(\lambda, C, d) * OPL$$

The scattering coefficient, $\mu_o(\lambda, C, d)$ is equal to the fraction of energy dispersed from a light beam per unit of distance traveled in a scattering medium, in cm^{-1} . For example, a liquid having $\mu = 1 cm^{-1}$ will scatter 90% of the energy out of a light beam over a distance of 1 cm, and corresponds to $S = 1 AU$. We note here that we have used the same units for both scattering and absorbance. Although this notation is not strictly rigorous, as will be illustrated further below, it can nonethe-

less be justified mathematically in the operating regime of the TruCell sensor. The scattering coefficient of pure water at $\lambda = 850 nm$ is less than $0.0013 cm^{-1}$ (primarily due to density fluctuations) and this is negated by zeroing in pure water during primary calibration. At a fixed wavelength and collection angle, for low particle concentrations, the scattering coefficient is proportional to particle concentration, C , namely, $\mu_o(\lambda, C, d) = \xi_{scatter}(\lambda) * C$. The scattering coefficient can also be related to the number density of particles, N , through the scattering cross section, $\sigma_{scatter}$, namely:

$$\mu(\lambda, N) = \sigma_{scatter}(\lambda) * N.$$

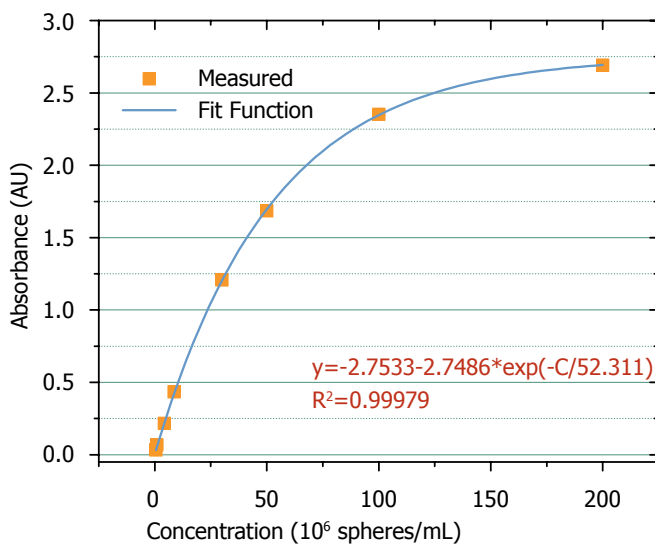
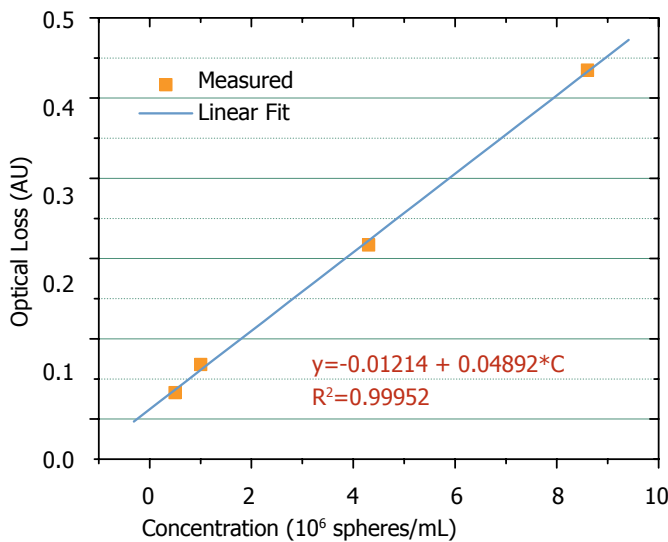
Figure 5 illustrates the dependence of scattering loss on concentration, for 3 micron diameter polystyrene spheres in water. The measurements were made using a fixed path length of 10 mm, a 20 degree forward-scattering collection angle, and an excitation wavelength of 850 nm. In Figure 5a, the scattering loss was measured for total optical losses of less than 0.5 AU, and the relationship between optical loss and concentration is indeed linear. In Figure 5b, the scattering loss was measured for total optical losses significantly exceeding 1.0 AU, and the relationship is highly non-linear. We note that for TruCell the scattering loss saturates exponentially as a function of particle concentration:

$$AU(C) = A_1 * [1 - \exp\{-C/C_0\}] + A_0$$

This deviation from linearity (versus absorbance) indicates that the light becomes heavily forward scattered in the presence of a high concentration of scattering centers. The light is highly dispersed, but, despite multiple scattering events, still reaches the detector. Further details can be found in the Appendix of the technical note entitled “OD vs. AU in Fermentation Cell Density.”

Because of the many combinations of particle size, shape, and color, similar scattering coefficient readings can be obtained from samples containing physically distinct particles. Furthermore, the scattering coefficient depends strongly on the size distribution of the particles. In most real-world applications, there will be different particles having different sizes, indices of refraction, and concentrations. The overall scattering coefficient will be a composite function of all these parameters.

Figure 5 a and b Scattering loss measured as a function of particle concentration at 850 nm over a 10 mm path length. The media consisted of 3 micron polystyrene spheres dissolved in water. **a** Optical losses lower than 1 AU, and **b** optical losses exceeding 1 AU.





Other Sources of Non-Linearity in Optical Measurements

In addition to absorption and scattering losses, an optical transmission sensor can encounter other types of optical losses which are typically non-linear, and may distort the measurement. We note that a laser-based sensor having optical windows whose index of refraction is matched to the bioreactor medium will avoid most of these potential pitfalls. However, a lamp or LED-based sensor having sapphire windows will not perform as well.

Radiation Source Wavelength

Typical cell density measurements are made using the 500 to 700 nm wavelength range. Typical in-line absorbance measurements are made using 850 nm light. Although at first glance the difference in wavelength does not seem that significant, one will recall that scattering mechanisms depend strongly on wavelength, and that this relationship is non-linear. If the lamp or LED-based cell density sensor has any variation in its operating wavelength, a different result may be obtained. Note that laser sources show excellent wavelength stability, and therefore this source of error will not occur with TruCell sensors

Radiation Source Bandwidth

A typical lamp or LED light source used to measure absorbance can have a bandwidth of 20 to 50 nm, and therefore produce a measurement that is a convolution of the optical response over a wide variety of wavelengths. Such a response can often lead to non-linear sensor performance. A laser-based absorbance sensor, however, will have a bandwidth of 0.5 nm to 3 nm, and should produce highly consistent and accurate results.

Homogeneity of Medium

When absorbance measurements are being made in real-time inside the reactor, the measurement is subject to the effects of agitation and sparging, in that the liquid medium can contain

bubbles, floating detritus and suspended cells or bacteria. These dynamic effects present during in-reactor measurements may further add to the scattering and absorbance losses of the cell themselves, and confound the optical loss measurement. Care must be taken to ensure that the TruCell probe active measurement volume is placed as far away from the sparger as possible yet is completely covered by the cell medium, to avoid affecting the measurement.

Optical Source Divergence and Effective Path-Length

Under most circumstances, a well collimated optical beam should provide unambiguous measurement results. Specifically, using a collimated beam, the optical path length (OPL) to the detector will be well defined and only in the case of very high optical loss will the analysis deviate from direct application of Beer's Law. However, for incoherent radiation sources such as a lamps or LEDs used in typical cell density sensors, the optical beam will generally not be perfectly collimated. When a highly divergence source is used, the light reaching the detector will be comprised of a multitude of different effective optical path lengths. This results in an average OPL thereby making the measurement of the OPL in a NIST-traceable manner more difficult.

For a laser-based absorbance sensor, the light will be well collimated and therefore the OPL will closely match the sample physical length. In addition, for a Teflon-based sensor, the optical windows will generally be well matched to the sample in index of refraction, further eliminating possible complications. The laser-based sensor will also have a greater manufacturing tolerance and ease of calibration, and thereby correspondingly lower cost.

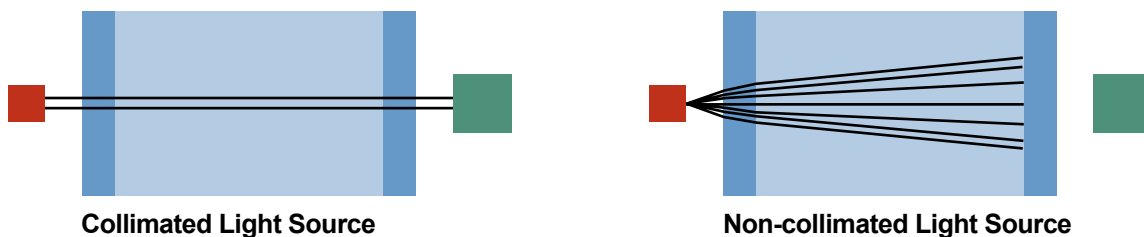


Figure 6 Difference in optical path length between a collimated (left) and non-collimated (right)



Examples of Optical Measurements

For TruCell measurements, we expect that the primary optical loss mechanisms will be scattering and absorbance. The most general fitting function of measured optical loss versus cell density will typically have the mathematical form:

$$AU(C) = A_0 + A_1 * C + A_2 * (1 - \exp[-C/C_0]),$$

where A_0 is the offset, A_1 is the absorption coefficient, A_2 is the effective scattering coefficient, and C_0 is the scattering constant. Such fitting functions will typically be observed for bacterial applications, where the concentration can become very high, so that the scattering loss will dominate. For mammalian cell culture

applications where concentrations are low, the optical losses measured will be much lower, and a linear approximation to the fitting function will normally be sufficient:

$$AU(C) \approx A_0 + A_{\text{slope}} * C$$

where A_0 is the offset and $A_{\text{slope}} \approx A_1 + A_2/C_0$.

Figure 7 shows the response of a TruCell sensor to high concentrations of yeast slurry used in pitching. The optical loss becomes high at high yeast concentrations, and comprises both absorption and scattering loss mechanisms. The measurement also has a significant initial offset. Note the close fit of the measured data to the general mathematical form for optical loss.

Figure 8 shows the response of a TruCell sensor to typical concentrations of Chinese hamster ovary (CHO) mammalian cells during a cell culture. The optical loss remains low, and the fit function can be approximated by a line. The measurement only has a small initial offset. Note also the close fit of the measured data to the linearized mathematical form for optical loss.

Figure 7
Response of TruCell AU versus Yeast Concentration

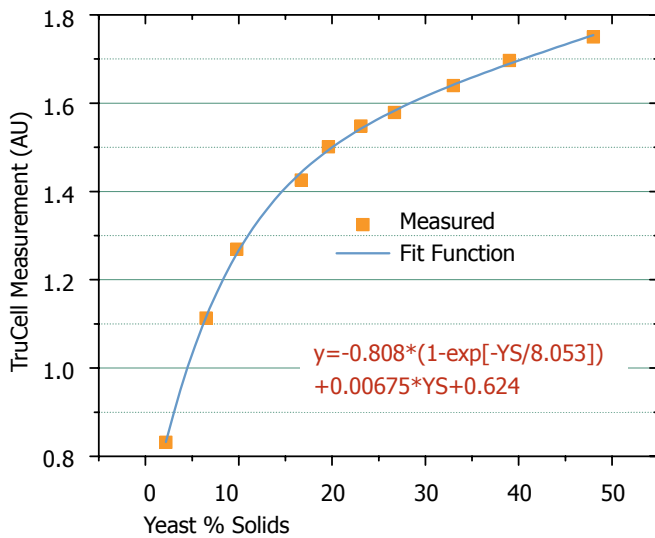
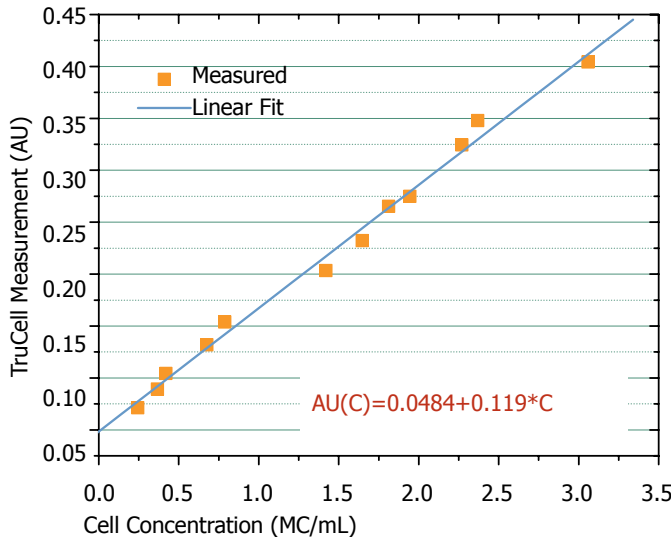


Figure 8
Response of TruCell AU versus CHO cell Concentration



In some cases, however, where the sensor is close to a sparger, bubbles may disrupt the measurement by producing additional scattering losses that are no longer correlated.



Appendix: Mathematical Details of Scattering Processes

Rayleigh Scattering

Rayleigh scattering is defined “is the scattering of light by particles much smaller than the wavelength of the light. [...] The amount of Rayleigh scattering that occurs to a beam of light is dependent upon the size of the particles and the wavelength of the light; in particular, the scattering coefficient, and hence the intensity of the scattered light, varies inversely with the fourth power of the wavelength, a relation known as the Rayleigh law. [...] The angular distribution of Rayleigh scattering [...] is symmetric in the plane normal to the incident direction of the light, and so the forward scatter equals the backwards scatter. Integrating over the sphere surrounding the particle gives the Rayleigh scattering cross section, σ_s :

$$\sigma_s = \frac{2\pi^5}{3} \frac{d^6}{\lambda^4} \left[\frac{n^2-1}{n^2+2} \right]^2$$

where λ is the wavelength of the light, n is the refractive index of the particle, and d is the diameter of the particle. The Rayleigh scattering coefficient for a group of scattering particles is the number of particles per unit volume N times the cross-section, σ_s . [...]”¹

Mie Scattering

For scattering by particles similar to or larger than the wavelength of the light, the behavior follows Mie scattering. “In contrast to Rayleigh scattering, Mie theory embraces all possible ratios of [particle] diameter to wavelength. It assumes a homogeneous, isotropic, and optically linear irradiated by an infinitely extending plane wave [of radiation]. [...] A further application is optical particle characterization. Mie theory is also important for understanding the appearance of common materials like milk, biological tissue, and latex paint.”² Mie theory is relatively complex and details will not be given here. The interested reader is referred to the following references:

1. Stratton: Electromagnetic Theory, New York: McGraw-Hill, 1941.
2. H. C. van de Hulst: Light scattering by small particles, New York, Dover, 1981.
3. F. Bohren, D. R. Huffmann: Absorption and scattering of light by small particles. New York, Wiley-Interscience, 1983.

Table 1 Size ranges for common “particles” found in bioreactors

Type of Particle	Size (micron)
Virus	0.05 – 0.4 (Mimivirus)
Bacteria	0.15 (Microplasma), 2.0 (E. Coli), 500 (Thiomargarita)
Eukaryotic Animal Cells	10 – 30
Eukaryotic Animal Cells	10 – 100
Bubble	100 – 25,000
Detritus	0.1 – 100

Table 1 shows the size ranges of bacteria, cells, bubbles, and detritus commonly found in bioreactors. For 500 to 850 nm light, we see that Mie scattering theory must be applied, because the wavelength is comparable in size or smaller than most of the particulates.

A Mie scattering calculator can be found at: http://omlc.ogi.edu/calc/mie_calc.html. Figure 9 shows the Mie scattering pattern as a function of angle for two cell models³: one with mitochondria and one with just a nucleus. We note

that most of the scatter is forward scatter, with scatter at 45 degrees being reduced by four orders or magnitude, and side scatter being further reduced by another order of magnitude.

In conclusion, turbid media with heavy scattering losses do not follow the Beer-Lambert law and if the scattering losses exceed the absorbance losses, this can lead to a non-linear relationship between off-line OD and in-reactor absorbance measurement results.

¹ http://en.wikipedia.org/wiki/Rayleigh_scattering

² http://en.wikipedia.org/wiki/Mie_scattering

³ A. Dunn, R. Richards-Kortum, IEEE J. Quant. Elec., vol. 2, no. 4, p. 898 - 905 (1996).

Figure 9
Mie scattering patterns for a cell with only nucleus and cytoplasm (solid), and a cell with mitochondria (dotted)³

