Technical Note

TruCell

OD^{600} vs. AU Measurements in Fermentation Cell Density

This technical note explains the relationship between two units commonly used in cell density measurements: namely, optical density units (OD or OD^{600}) and the basic absorbance units (AU). Both of these units can be correlated to the physical cell density (PCD) of the sample, such as dry cell weight (DCW) or cell concentration (MM cells/mL).

Off-Line Biomass Measurements

Biomass concentration is one of the most critically needed measurements in fermentation and cell culture studies. Biomass concentration is typically measured by one of two off-line methods, wherein a small sample of the fermentation broth is extracted from the reactor:

- Total Cell Density (TCD): Counting the number of cells within a known volume of sample. In this process, number of cells can be counted either by successively diluting the original sample and plating on a Petri dish, with the help of a microscope and a counting chamber, or with an automated cell counter such as a Coulter counter or a flow cytometer. The plating method detects viable cells; whereas, the automated cell counters detect the total number of cells. This value is typically reported in units of MM cells/mL (millions of cells/milliliter).
- Cell Weight (CW), which can be either dry cell weight (DCW) or wet cell weight (WCW): Weighing the cells in a known volume of sample. In this process, the cells in a sample are separated from the broth and weighed while they are wet, or the cells may be thoroughly dried before weighing. The dry weight measurement usually gives a much more consistent result than the wet weight. This value is typically reported in units of g/L (grams/liter).

Both of these off-line methods either require the availability of expensive equipment or the substantial investment of time. Moreover, both of these methods can be difficult and unreliable. Both are also susceptible to operator error in dilution or evaluation/counting. At a minimum, neither method provides real-time information for process control.

For example, dry/wet weight methods and automated counting equipment fail completely if the broth contains other insoluble particulate matter, which is often the case in a practical bioreactor. Furthermore, CW methods cannot distinguish the viable cells from the dead ones. On the other hand, the standard plate count can detect viable cells among other particulate matters. However, the method requires elaborate preparations, and it takes 24-48 hours for the cells to be incubated and counted. Consequently, the utility of direct plate count is limited for feedback control of a fermentation process. Plating TCD methods are therefore mainly used by industry to countercheck other, less labor-intensive measurements, such as optical density.

In most applications, the simplest off-line method for estimating cell density is to monitor the optical density (OD) of the sample. Optical density and cell density readings can be related by a set of calibration curves that are generated from several bioreactor runs. Once OD measurements are mapped to biomass values, OD readings can provide useful feedback immediately. Typically, OD measurements are made once per shift during a fermentation or cell culture run, and provide information for feedback control. Ideally, these measurements should be performed in real-time and in the bioreactor.

Finesse, LLC
3350 Scott Boulevard #1
Santa Clara, CA 95054

9351 Irvine Boulevard
Irvine, CA 92618

800-598-9515
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Optical Density (OD)

For biological samples, the optical properties are often reported in terms of optical density (OD). The OD of a sample is measured in a 10 mm cuvette at a visible wavelength using a spectrophotometer. The sample is diluted until its total optical loss (absorption and scattering), OL, falls within the spectrometer's linear regime, namely, OL < 1.0 AU (where AU = absorbance units). The optical loss measured by the spectrophotometer is then scaled by the dilution factor to obtain the OD value. At a given measurement wavelength, λ, the OD value is given by the following equation:

$OD^{\lambda} = OL(\lambda) * F$
where F = Dilution factor required for the sample $OL(\lambda) < 1.0$ AU

The dilution factor can be quite high, and the dilution errors can often lead to significant operator error in the OD measurement. In fermentation applications, OD measurements are made off-line on a sample extracted from the bioreactor. OD is typically measured at wavelengths in the 500 to 700 nm wavelength range, and the measurement wavelength is noted as OD^{λ} (e.g., OD^{600} for λ = 600 nm).

For example, an OD measurement of a typical fermentation broth sample would proceed as follows:

1. A sample is extracted from the bioreactor into a 10 mm cuvette..
2. The lab spectrophotometer is set to read OL at 600 nm.
3. With no dilution, the sample OL reads 2.75 AU.
4. The sample is diluted twice for a total dilution factor of 100.
5. The diluted sample OL is read again, and the result is 0.459 AU.
6. The reported OD600 value is 0.459 x 100 = 46.

It is important to note that a linear relationship between OD and biomass concentration does not always apply when the optical losses become significant (OL > 1.0 AU). Why? Even at visible wavelengths, the total optical loss of a bioreactor sample comprises both absorption and scattering losses. At high biomass concentrations, the optical losses become dominated by scattering losses, and the spectrophotometer response to scattering losses saturates. For more details, please refer to the technical note entitled "Optical Measurements of Cell Density". Therefore, the OD value eventually saturates as the biomass concentration increases.

The Relationship Between Optical Density (OD) and Optical Loss (OL)

For real-time bioreactor control, the off-line OD measurements are gradually being replaced with real-time, in-reactor optical loss (OL) measurements. In general, although OD and AU are both a measure of optical loss, the relationship between OD and AU is not linear for two main reasons:

1. In-line sensors that measure OL use near-infrared (850 nm) wavelength, whereas traditional OD measurements use a visible wavelength such as 600 nm. Biological substances generally do not absorb strongly in the near-infrared, so the optical loss is dominated by scattering. The popular OD^{600} measurement occurs at 600 nm, however, and will include both scattering and optical absorption losses.
2. The design of the instrument or sensor used for the measurements is different. OD measurements are made using a spectrophotometer, whereas in-line sensors such as TruCell use a laser. The properties of the light source and collection optics geometries are quite different, and can result in a non-linear relationship even when the measurement wavelength used is the same.

In the appendix, we discuss the various contributors to this non-linear relationship. We also show examples that illustrate how the relationship between laboratory spectrophotometer and an in-line sensor can change depending on the type of sample being measured. We demonstrate that for our cell density probe, a single equation can be used to describe all forms of the OD and OL relationship.

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Santa Clara, CA 95054

9351 Irvine Boulevard
Irvine, CA 92618

800-598-9515
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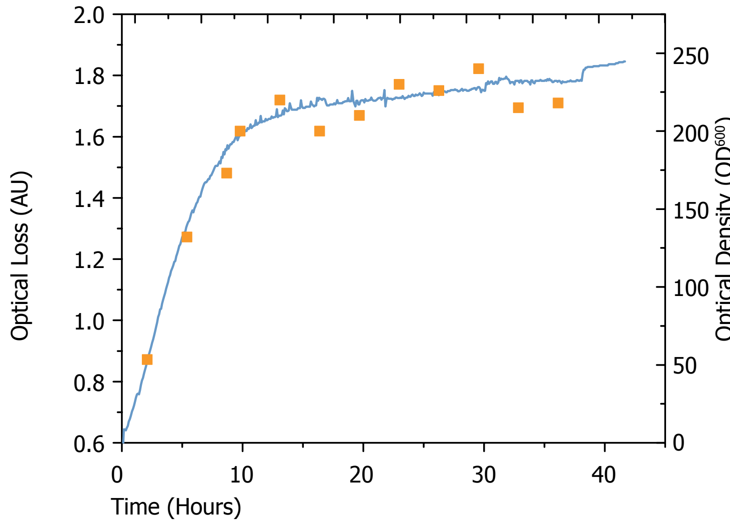


Figure 1
Reference fermentation run where both real-time OL and off-line lab OD^{600} values were measured simultaneously.

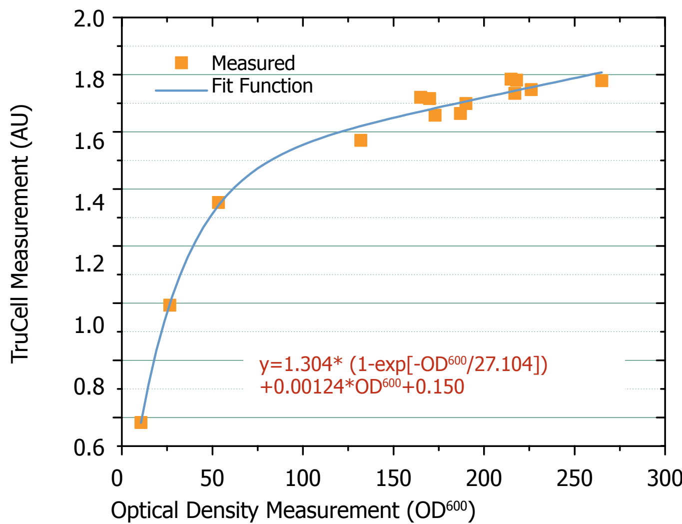
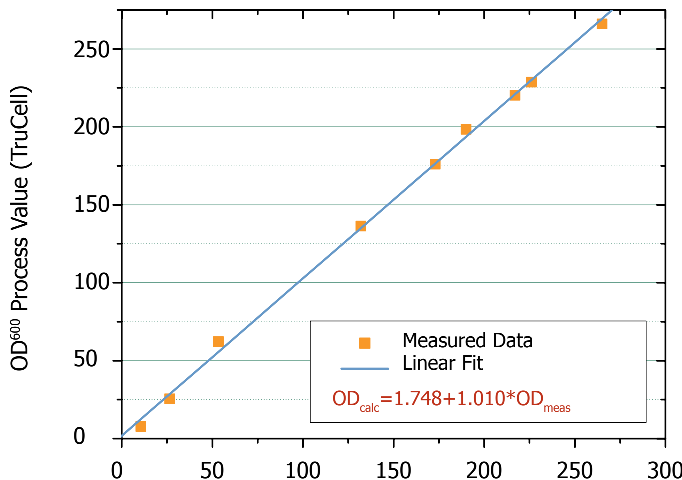


Figure 2
Calibration curve example showing the non-linear relationship between in-line OL and off-line OD measurements.



In-reactor Optical Loss (OL) Measurements

In this section, we will show how in-line, real-time OL measurements provide a surrogate for off-line, laboratory OD measurements. In most applications, a calibration run must be performed to map the relationship between OD and OL. Once this calibration is determined, the OL measurement can be used to compute an effective OD value. Because OL measurements are real-time and the signal is available immediately, OL measurements can be used for real-time process control.

The TruCell sensor measures the real-time OL within the probe's measurement gap. This gap has a physical optical path length where the light passes directly through the liquid medium inside the bioreactor. TruCell reports the raw OL in AU units. TruCell can also be calibrated to directly report the "effective" real-time process value: OD, physical cell density, dry cell weight, etc.

We present an example of using the TruCell sensor to perform a calibration between off-line OD^{600} measurements and raw in-line OL readings. In a subsequent process run, the calibrated TruCell OD^{600} values are in excellent agreement with the actual measured OD600 values.

In the first step, simultaneous in-line OL and off-line OD^{600} measurements were taken as a function of time during a "reference" fermentation growth run (Figure 1).

Once both OL and OD values for a specific process were measured, a calibration curve relating OL and OD^{600} was established (figure 2). The calibration curve equation was entered into TruCell, so that the sensor reported and output a "process value" for convenience. In our example, the TruCell output could be scaled to the OD^{600} value and the OD600 output range was set from 0 to 12.

Once the curve fit was activated, the TruCell process output value had a linear correlation to laboratory cell density values, as shown in figure 3.

TruCell

Appendix: Sources of Non-Linearity in the Relationship Between Optical Loss and Optical Density Measurement

The relationship between optical loss (OL) and optical density (OD) is often non-linear because the measured response depends on both absorption and scattering losses, whose relative contributions to the total optical loss are highly dependent on the measurement instrument and wavelength used. Specifically:

- The measurement at 600 nm (used for OD) is dominated by absorption, whereas at 850 nm (used for OL), it is dominated by scattering
- Because the light source emission profile and the geometry of the detection optics are different for a spectrophotometer (used for OD) and an in-line sensor (used for OL), the resulting distribution of light both in the sample and arriving at the detector differs as a function of scattering and measurement wavelength.

We provide example measurements in the appendix that show how OD and OL maintain a linear relationship for samples having only absorption and no scattering. We also provide examples where OD and OL have a non-linear relationship when the measurement wavelengths are different, or the dominant loss mechanism is a combination of absorption and scattering.

Scattering Losses

For biological samples, especially those related to fermentation or cell culture, there is a very high degree of non-linearity between the optical loss measurement and cell concentration, owing to the optical loss being dominated by scattering processes. The type of scattering process depends on the relative size of the particulate (e.g., bacteria, cells, bubbles, or detritus) and the wavelength of light used by the measurement. The two types of scattering losses, namely Rayleigh and Mie scattering, are discussed in detail in the “Cell Density Primer” technical notes.

Rayleigh scattering theory is used when the particles are much smaller than the wavelength of the light used in the measurement of optical loss. The Rayleigh scattering cross section, σs, varies inversely with the fourth power of the wavelength and proportionally to the sixth power of the particle diameter:

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

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 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.

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. […] 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 the details will not be given here.

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.

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)
Eukaryotic Animal Cells	10 – 30
Eukaryotic Animal Cells	10 – 100
Bubble	100 – 25,000
Detritus	0.1 – 100

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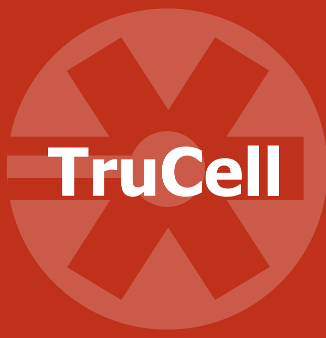


Measurements Systems: Spectrophotometer and TruCell

We consider a typical UV-VIS spectrophotometer used in making optical density measurement (Figure 4). It uses either a Deuterium (190 nm to 800 nm) lamp or a Tungsten (370 nm to 1100 nm) lamp as a light source, depending on the target measurement wavelength. The light source is broadband, incoherent, and highly divergent.

In the basic optical design, the source light is collimated using a lens, and passes through an electromechanical shutter/stray light filter (which can block UV light below 400 nm for visible wavelength measurements) before entering the sample. The sample is placed in a quartz, glass, or plastic cuvette (depending on the target wavelength). The light transmitted through the sample is focused by a lens onto the narrow aperture of a slit (having a 1 nm spectral width), and dispersed on a concave holographic grating. The resulting “fan” of light is detected using a linear array of photodiodes, which can contain up to 1024 elements. The mean sampling interval (or spectral resolution) of the detection system is about 0.9 nm from 190 nm to 1100 nm.

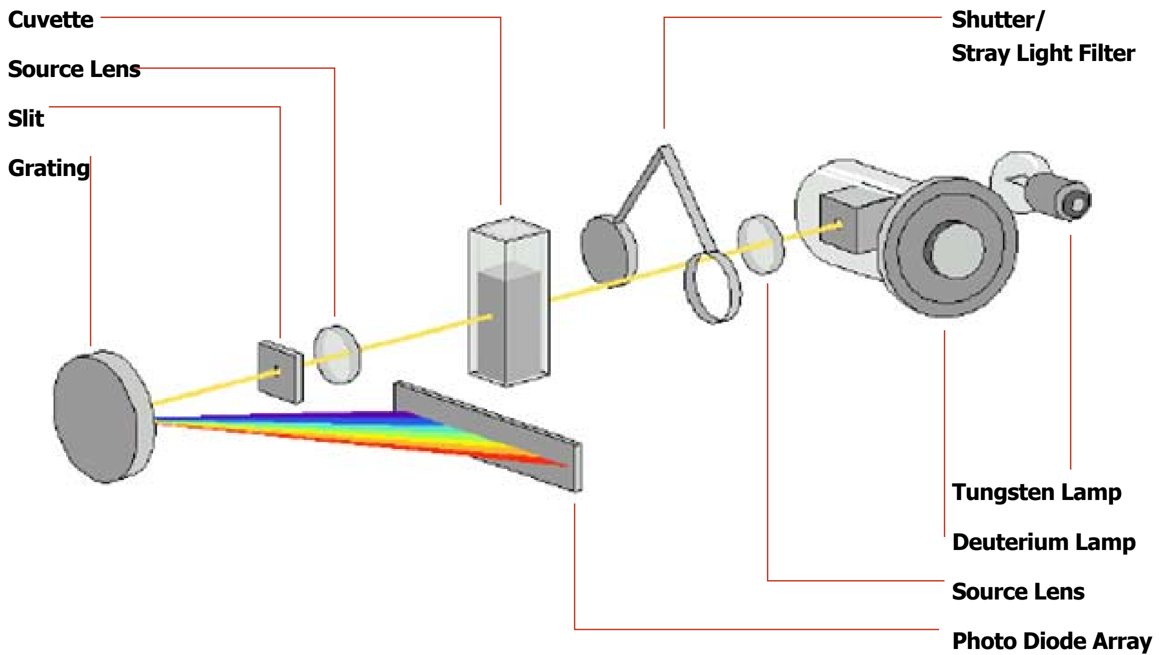


Figure 4 **Optical design of a typical UV-VIS spectrophotometer used in making optical density measurements**

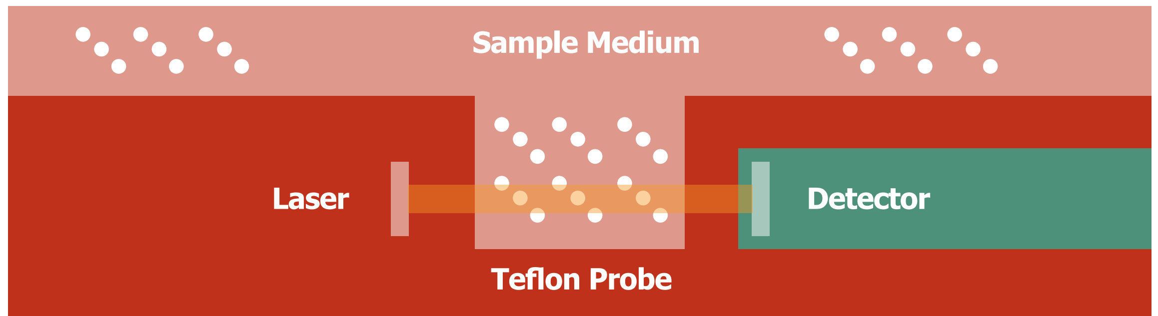
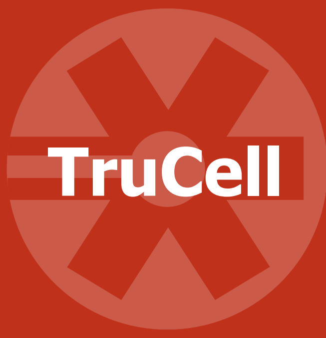


Figure 5 **Optical design of a TruCell probe used in making optical loss measurements**

Finesse, LLC
3350 Scott Boulevard #1
Santa Clara, CA 95054

9351 Irvine Boulevard
Irvine, CA 92618

800-598-9515
www.finesse-inc.com



The TruCell sensor, on the other hand, consists of a laser source that is directed through a Teflon window (Note that Teflon has very low optical loss at 850 nm) into the sample medium. After the light is transmitted through the sample, it again passes through a teflon window and is directly collected by a single photodetector. The laser light is monochromatic and narrowband, with a center wavelength at 850 nm. The laser light has a much lower divergence.

In addition to the light source and collection optics differences, the window materials can also contribute to the non-linear relationship between OD and OL measurements. TruCell windows are made of Teflon, which has an index of refraction at 600 nm of 1.34. Spectrophotometer cuvettes are made of glass, Pyrex, or quartz, which have an index of refraction at 600 nm of 1.52, 1.47, and 1.46, respectively. For a typical water-based solution, the index of refraction at 600 nm is 1.33, while that of cells is estimated to be about 1.35.

For incoherent light sources such as the lamps used in a spectrophotometer, the optical beam will not be perfectly collimated, so that the mismatch between the optical windows and the sample will produce a light path through the sample that differs from the physical sample length (i.e., the light beam will travel at an angle through the sample). For a laser-based absorbance sensor such as TruCell, however, the light should be well collimated and the windows well matched to sample in index of refraction, so that the optical path length should match the sample physical length. This difference in response can lead to further non-linearity between OD and OL measurements.

Finally, we should note competing in-line sensors use an LED light source and sapphire windows that have an index of refraction of 1.77 at 600 nm. These sensors should produce a response that is somewhat in between TruCell and a spectrophotometer. This means that these sensors will have a non-linear relationship with both TruCell OL readings and OD values.

OD and OL: Absorbance Only Example

According to the Beer Lambert law, the relationship between OL or OD and concentration is linear. We would therefore expect that a sample having only absorbance would produce a linear response from both a spectrophotometer and TruCell. Therefore, the relationship between OD and OL would also be linear. Furthermore, if the sample absorption remains within the linear dynamic range of the spectrophotometer, and the absorbance is relatively constant between 800 nm and 850 nm, we would expect an almost one-to-one mapping between the TruCell OL and spectrophotometer OD measurements.

As a model, we use a solution of copper sulfate. Copper sulfate has a well-known spectrum with an absorption peak at 810 nm (figure 6a) that is broad, and will have a similar response at both 800 nm and 850 nm. This absorption feature can be readily measured using a spectrophotometer for different copper sulfate concentrations (figure 6b). No dilutions are required for a 10 mm sample cuvette. From these measurements, the relationship between OD and concentration can readily be determined (figure 7). Similarly, the OL using a TruCell sensor can be measured (figure 7). Note that both of these relationships are linear. Consequently, it naturally follows that the relationship between OD and OL is also linear (figure 8) and has a nearly one-to-one mapping.

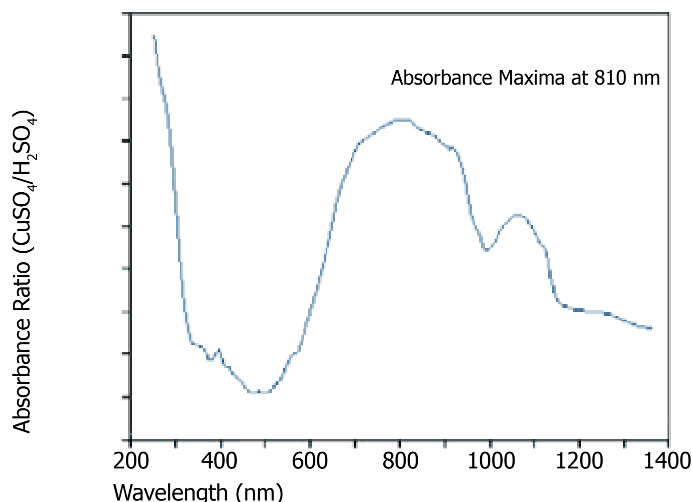


Figure 6 (a)
Spectrum of copper sulfate solution from a reference text

OD and OL: Scattering Only Example - Microspheres

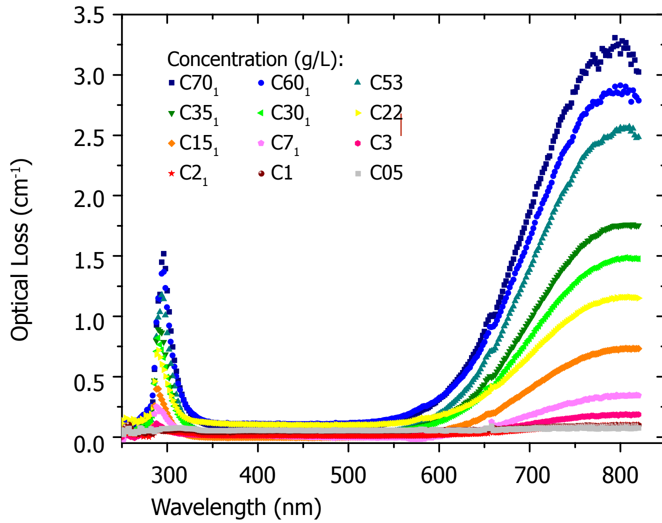


Figure 6 (b) spectra of different concentrations of copper sulfate solution using an Agilent spectrophotometer

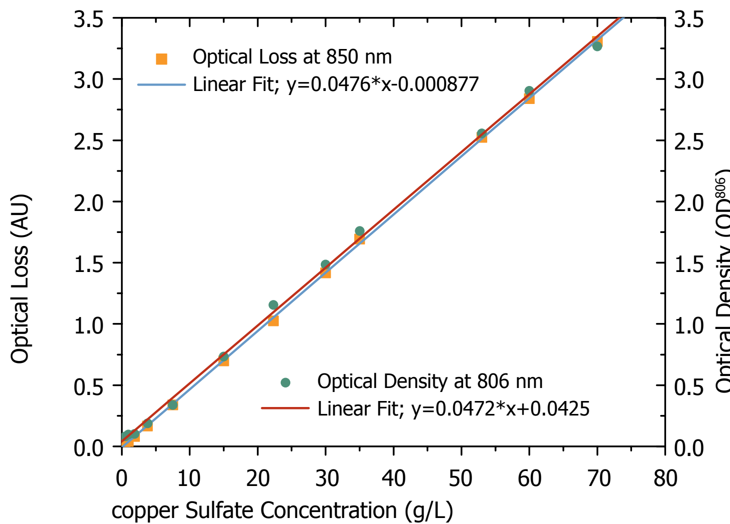


Figure 7 Linear relationship between copper sulfate solution and optical density or optical loss.

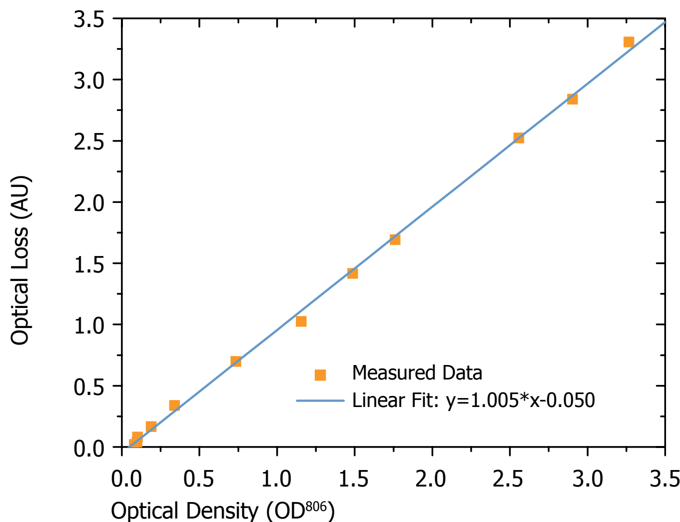


Figure 8 Linear relationship between optical density and optical loss for a series of different copper sulfate solutions.

The relationship between OL or OD and the concentration of particles that are non-absorbing will generally be non-linear. We would therefore expect that a sample having only scattering losses would produce a non-linear response from both a spectrophotometer and the TruCell sensor. The forward scattering distribution is expected to be uniform, so that the results should be relatively independent of the instrument light source and optics. Therefore, if the sample scattering loss remains within the linear dynamic range of the spectrophotometer, then we expect a linear mapping between the TruCell OL and spectrophotometer OD measurements at the same wavelength.

However, Mie theory predicts that the scattering loss will strongly depend on the measurement wavelength. Therefore, the relationship between OD and OL will be highly wavelength dependent, so that we expect a non-linear relationship between the TruCell OL and spectrophotometer OD measurements at a different wavelength.

As a model, we use a solution of 3 micron polystyrene spheres in water. These spheres are extremely uniform in size (only a 5% standard deviation). These spheres form a homogenous medium that has minimal absorption at near-infrared wavelengths, so that the optical losses from the medium are predominantly due to scattering. This scattering loss can be readily measured using a spectrophotometer for different concentrations of spheres (figure 9).

Without dilution, a concentration range of 5×10^5 spheres/mL to $5 * 10^7$ spheres/mL could be measured using the spectrophotometer and a 10 mm sample cuvette. For concentrations exceeding 5×10^7 spheres/mL, the optical loss exceeded the spectrometer's dynamic range. From these measurements, the relationship between OD and sphere concentration can readily be determined (figure 10). Similarly, the OL using a TruCell sensor was measured (figure 10). Note that the TruCell sensor did not saturate and was able to measure the entire sphere concentration range from 5×10^5 spheres/mL to $2 * 10^8$ spheres/mL.

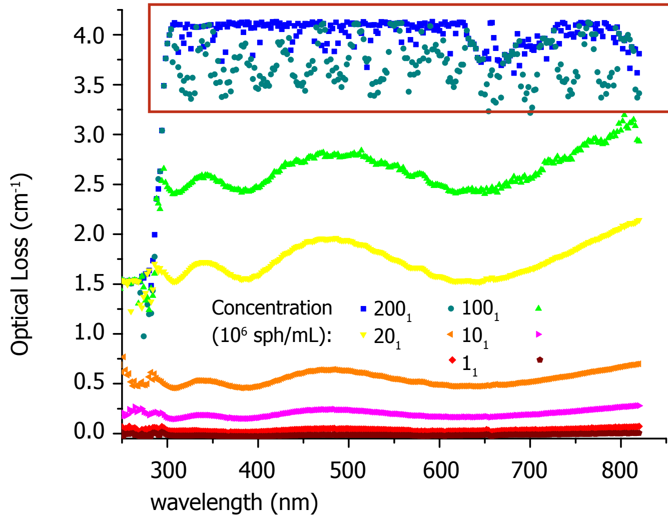


Figure 9
Spectra of different concentrations of 3 micron diameter polystyrene spheres in water, taken using an Agilent spectrophotometer

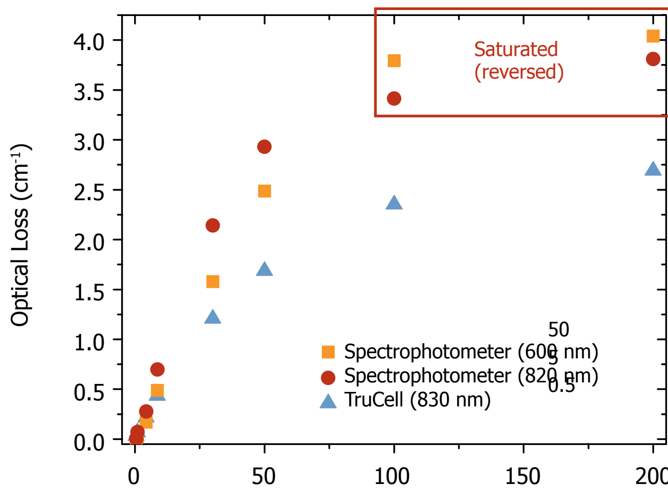


Figure 10
Non-linear relationship between the concentration of 3 micron diameter polystyrene spheres in water, and optical density (at two wavelengths) or optical loss.

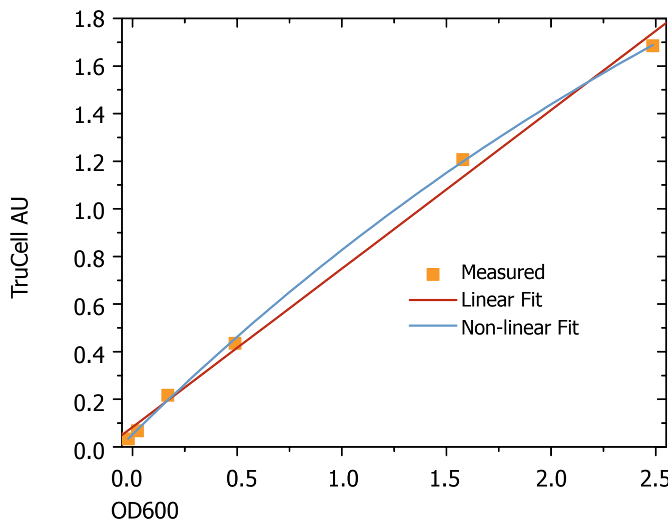


Figure 11 a
Non-linear relationship between optical density at 600 nm and optical loss at 830 nm

We observe that both the OD and OL relationship with sphere concentration is non-linear. Furthermore, we note that the saturation of the spectrometer leads to erroneous results, so that the two readings at the highest sphere concentrations must be ignored (or the sample re-measured using dilution).

From figure 10, we also note that the optical loss measured at 820 nm using the spectrophotometer exceeds that reported by the TruCell sensor by almost a factor of two. This difference illustrates the difference in the collection optics between the two instruments: the spectrometer has a narrower aperture.

Figure 11 shows the relationship between OD and OL when the wavelengths of TruCell and the spectrophotometer are (a) different, and (b) similar. We see that for approximately the same wavelength, the relationship is linear, although the slope between OL and OD is about 0.56 (i.e., the ratio of OD to OL is approximately two), which indicates that the TruCell has a higher collection efficiency than the spectrophotometer. For different wavelengths, however, the relationship is non-linear and can be expressed using the same equation as that used to correlate OL with OD for cells (see figure 2), but without the linear term. The linear term is absent, because the polystyrene spheres do not absorb in the visible and near-infrared.

OD and OL: Scattering and Absorption Example – Formazin

Finally, we investigate the relationship between OL or OD and sample concentration for a sample that produces both absorption and scattering losses, and that has a larger size variation (and therefore a much more complex scattering function). We would therefore expect that this sample will produce a non-linear response from both a spectrophotometer and TruCell. Owing to the non-homogeneity of the medium and the complicated form of the optical loss, the relationship between OD and OL would also be non-linear, even at the same measurement wavelength.

As a model, we use a solution of formazin. Formazin is a primary U.S. standard used for

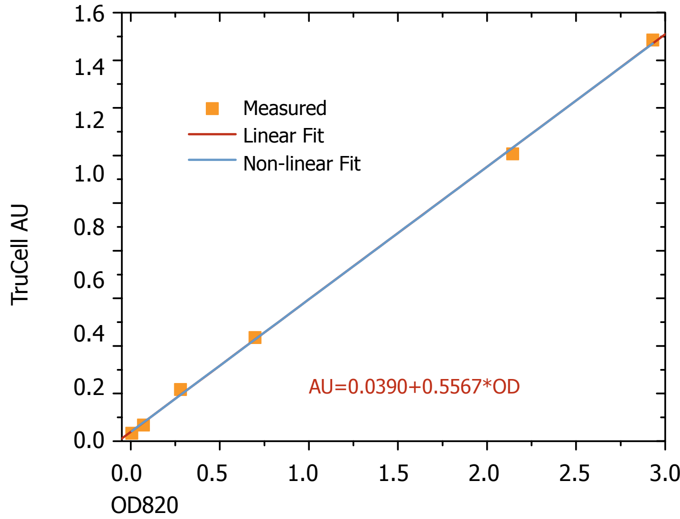


Figure 11 b
linear relationship between optical density at 800 nm and optical loss at 830 nm, for a series of different concentrations of 3 micron diameter polystyrene spheres in water.

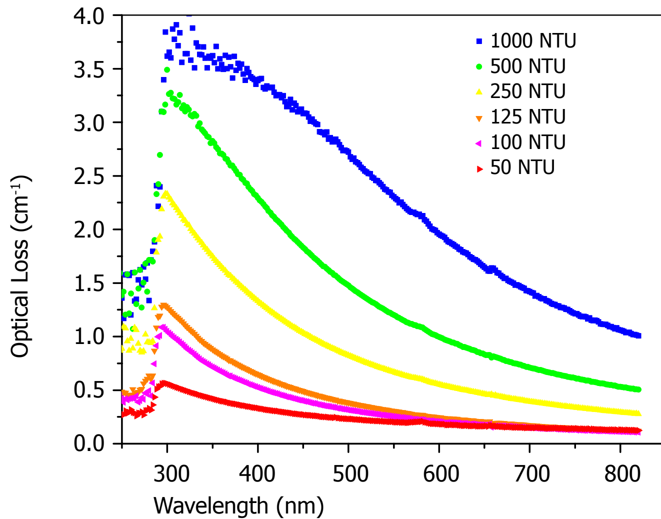


Figure 12
Spectra of different concentrations of formazin, taken using an Agilent spectrophotometer.

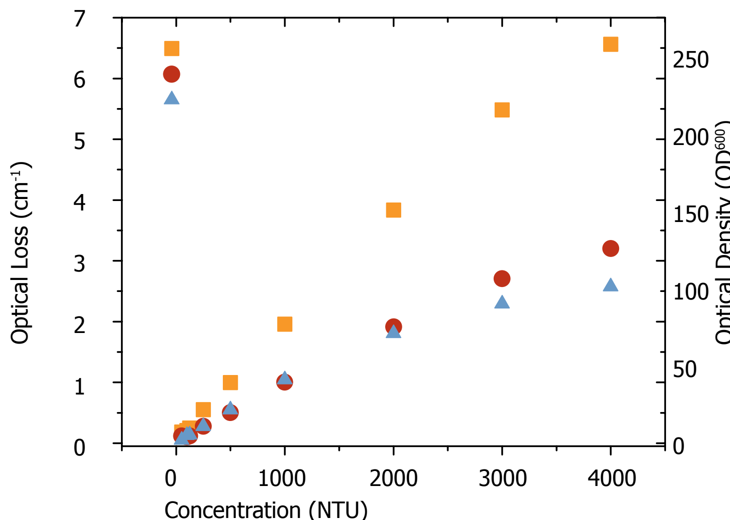


Figure 13
Non-linear relationship between the concentration of formazin, and optical density (at two wavelengths) or optical loss.

the calibration of turbidity meters. It has a median particle size of 1.5 micron, and a size distribution having a 40% variation. The particles can take many different shapes, from spherical to oblong to random. Figure 12 illustrates the spectral dependence of different concentrations of formazin, which range from 50 NTU to 1000 NTU. No dilutions were required for a 10 mm sample cuvette, although the spectrometer response appeared to saturate for the 1000 NTU solution. The spectrophotometer response did saturate for higher formazin concentrations.

Note that the optical loss decreases as a function of wavelength. The optical loss comprises both absorption and scattering. From the spectral measurements, the relationship between OD (at 600 nm and 800 nm) and formazin concentration were determined (figure 13). Similarly, the OL using a TruCell sensor was measured (figure 13). Note that all of these relationships are non-linear, with the OD600 response exhibiting the highest absorption, and hence "closest" approximation to linearity. Note also that the TruCell and spectrophotometer DO800 measurements were in reasonable agreement, although the spectrometer again measured a slightly higher optical loss owing to its narrow slit.

From Figure 14, we conclude that both relationships between OL and OD600 or OD800 are non-linear. The same functional form, however, can be used to describe both.

From these examples, we can conclude that there always exists a relationship between OL and OD, which can be described by the following equation:

$$OL(OD) = A_0 + A_1 * OD + A_2 * [1-\exp(-OD/A_3)]$$

Where A_0 corresponds to an instrument offset, A_1 corresponds to an absorption term, and A_2, A_3 depending on the scattering function. This function can also be approximated using a third order polynomial that can be programmed into TruCell:

$$OL(OD) \approx A_0 + (A_1 + A_2/A_3) * OD - A_2/A_3^2 * OD^2 + A_2/A_3^3 * OD^3$$

For most cell culture applications, the total optical loss will remain fairly small, even at the end of the growth run, so that the polynomial approximation will be sufficient. In many cases, even the simple linear approximation will work quite well.

Figure 14 a
Non-linear relationship between optical density at 600 nm and optical loss at 830 nm

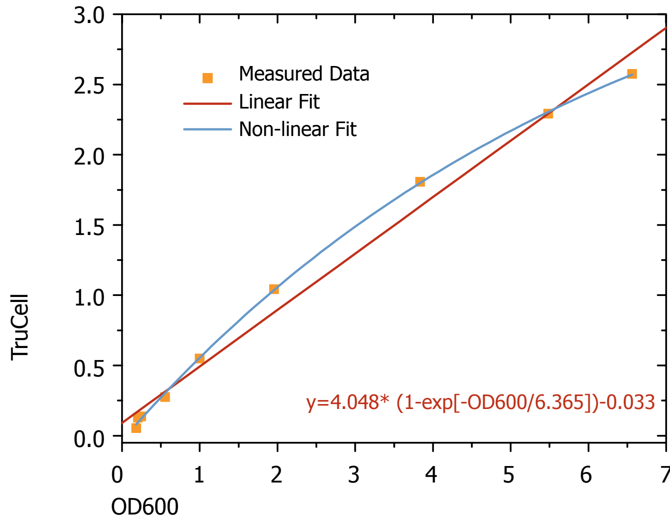
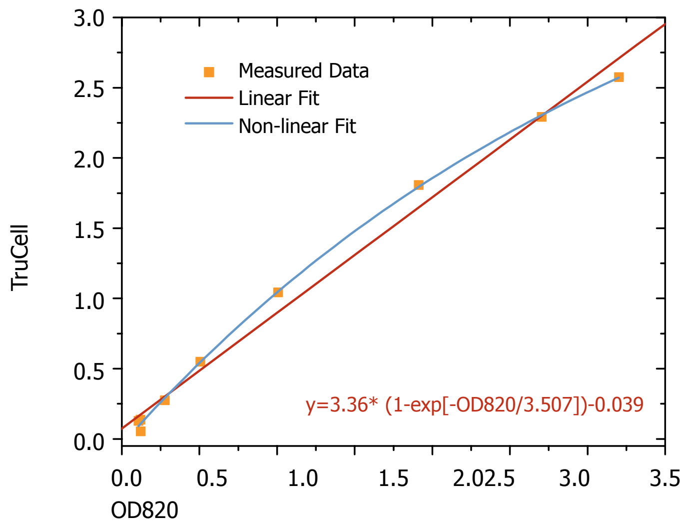


Figure 14 b
linear relationship between optical density at 800 nm and optical loss at 830 nm, for a series of different concentrations of formazin.



For bacterial fermentation applications, however, the total optical loss may become significant, so that the full polynomial approximation, or the full exponential equation will be required. In these cases, the relationship between OD, AU and the cell measurement parameters (such as dry cell weight) will be non-linear, and a calibration run will be required. In some cases, TruCell will need to be zeroed in the actual medium, in order to account for process variations. However, in all cases, once the relationship between the TruCell AU reading and the process variable is established, TruCell will measure and report it with good precision and repeatability.

We have demonstrated that the relationship between concentration and optical density or optical loss is non-linear for both a spectrophotometer and TruCell, respectively, because it is dominated by scattering (rather than absorption), and the geometry of the measurement systems is such that its response is non-linear. We have also shown that a single equation can be used to describe this non-linear relationship, and be used in calibration, to produce an effective linear response.

Finesse, LLC
3350 Scott Boulevard #1
Santa Clara, CA 95054

9351 Irvine Boulevard
Irvine, CA 92618

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