Dive deep into Stunner's light scattering

Introduction

Seeing the full biophysical picture of a biologics or gene therapy sample requires knowing more than just concentration – you need size, molecular weight, and to check for aggregation. Stunner (Figure 1) takes characterization to the next level by combining high speed UV/Vis spectroscopy with rotating angle dynamic light scattering (RADLS). RADLS upgrades classic DLS by rotating the DLS-module through multiple angles to get a unique set of data on every sample. RADLS combines the size data from those various angles into one angle independent Z-average hydrodynamic diameter so you get the most accurate size and catch even the tiniest bit of aggregation. Capturing data from multiple angles gives Stunner everything it needs for incredibly powerful sizing analysis.

This technical note describes in more detail the fundamentals of DLS, how Stunner leverages state-of-the-art optics to achieve RADLS and how Stunner fits everything together to get it done on 2 µL of sample in under 2 minutes.

For more information on how Stunner performs UV/Vis spectrophotometry, check out the our UV/Vis Spectroscopy Tech Page and for even more details our Tech Note "A look under the hood of Lunatic" – Stunner performs UV/Vis measurements the same way.

Fundamentals of light scattering

There are two major types of light scattering that are key to understand: Rayleigh (Figure 2) and Mie scattering (Figure 3).

Rayleigh scattering occurs when light interacts with particles that are much smaller than the wavelength of the light. That results in light scattering of equal intensity at all angles.
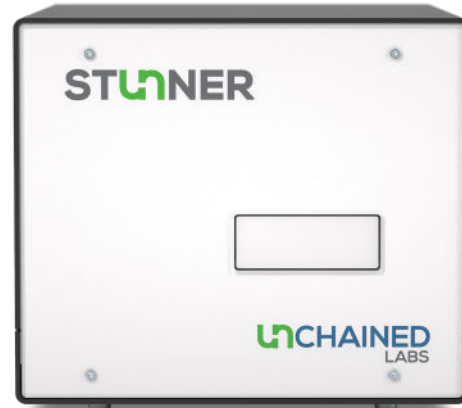

Figure 1: The ultimate quantification and sizing tool.

Key characteristics of Rayleigh scattering:

Particle Size: Rayleigh scattering occurs when particles are much smaller than the wavelength of light, typically less than 1/10th of the wavelength.

Scattering Intensity: The intensity of scattered light varies inversely with the fourth power of the wavelength $I \propto \lambda^{-4}$.

Angular Dependence: Rayleigh scattering is isotropic, meaning it occurs equally in all directions.

Mathematical Description: The intensity of Rayleigh scattering is given by the Rayleigh scattering formula:

Equation 1
$$I = I_0 \frac{8\pi^4 R^6}{3\lambda^4} \left(\frac{n^2-1}{n^2+2}\right)^2$$

Here, I_0 is the original light intensity, R is the radius of the particle, λ is the wavelength of light, and n is the refractive index of the particle.

Mie scattering describes light scattering by particles that are similar in size or larger than the wavelength of light. This type of scattering is especially relevant in understanding the optical properties of larger viral vectors, lipid nanoparticles, aggregates, and other colloidal systems.

Rayleigh scattering (isotropic)

- ● Particle
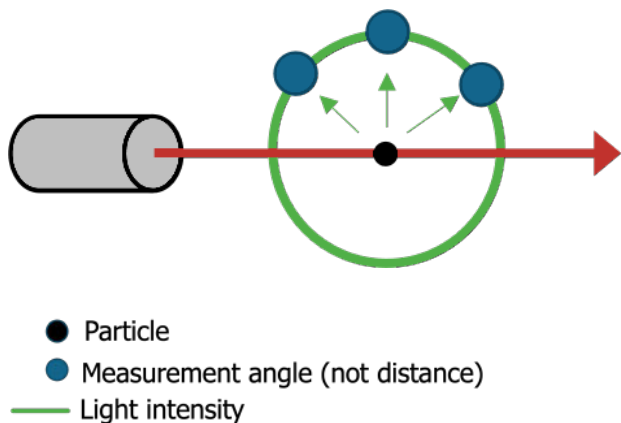- ● Measurement angle (not distance)
- ─ Light intensity

Figure 2: Principle of Rayleigh scattering. Rayleigh scattering occurs when light interacts with particles that are much smaller than the wavelength of the light. That results in light scattering of equal intensity at all angles.

Mie scattering (anisotropic)

- ● Particle
- ● Measurement angle (not distance)
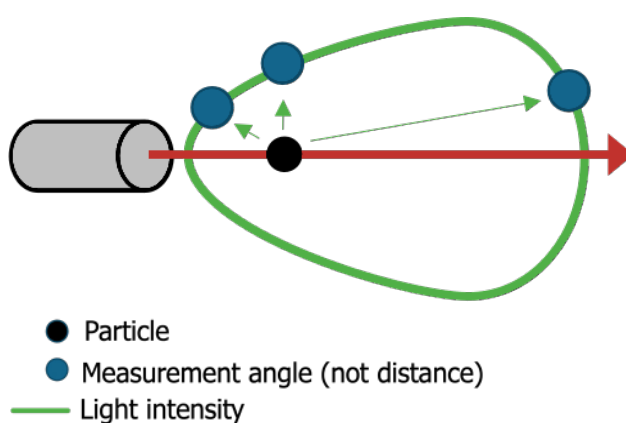- ─ Light intensity

Figure 3: Principle of Mie scattering. Mie scattering describes light scattering by particles that are similar in size or larger than the wavelength of light. This type of scattering is especially relevant in understanding the optical properties of larger viral vectors, lipid nanoparticles, aggregates, and other colloidal systems.

Key characteristics of Mie scattering:

Particle Size: Mie scattering is significant for particles with sizes comparable to or larger than the wavelength of light.

Scattering Intensity: The intensity and pattern of Mie scattering has a complex relationship to light wavelength, particle size, composition, and refractive index of the particles relative to the surrounding medium.

Angular Dependence: Mie scattering shows a more complex angular distribution compared to Rayleigh scattering, with varying intensities at different angles. Generally, Mie scattering is more intense in forward angles (angles less than 90°).

Mathematical Description: Mie theory provides a comprehensive solution for light scattering by spherical particles. The mathematical formulas are complex and require computational methods for practical application.

Key takeaway

The key difference lies in the size of the particles relative to the wavelength of light. Rayleigh scattering is for smaller particles and shows isotropic, wavelength-dependent scattering. Mie scattering applies to larger particles and results in scattering patterns where the angle between the light source and the detector matters a lot.

How does classic dynamic light scattering (DLS) work?

Shine a laser on a solution of particles and you'll get plenty of light scattering out from the particles in solution. DLS tells you a lot about the size of the particles by measuring how rapidly that scattered light changes over time (Figure 4). Since small particles zip around quickly, the intensity of light changes quickly. Vice versa for larger ones: as they are slower to move around, the intensity of light scattered by them changes more slowly. Analyzing whether light intensity is changing fast or slowly – that's the secret sauce of DLS.
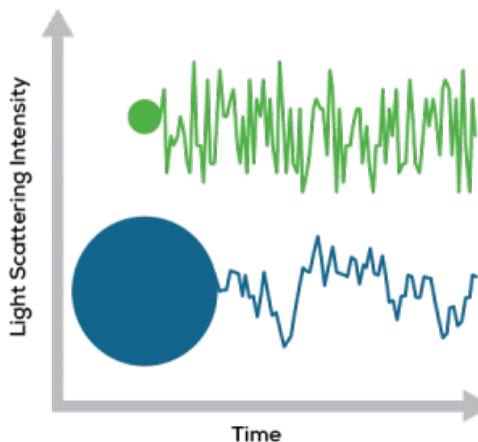

Figure 4: Light scattering intensity changes differently over time for differently sized particles.

Here's how to think about analyzing light scattering data for DLS: pick a point in time – now jump forward a microsecond. Odds are good most particles haven't moved around yet – so the light scattering hasn't changed and the data from time zero and a microsecond later are about the same. In other words, they have a high correlation.

Now instead of a microsecond, jump forward a full second. Most particles will be in totally different spots – and you now have zero correlation in the data between your starting point and your jump one second later. Graph these correlation values for a range of jumps of different durations and you get a Correlation Function (Figure 5). How quickly particles go from high correlation to zero correlation tells you their average size. This is also why DLS is sometimes called photon correlation spectroscopy (PCS).
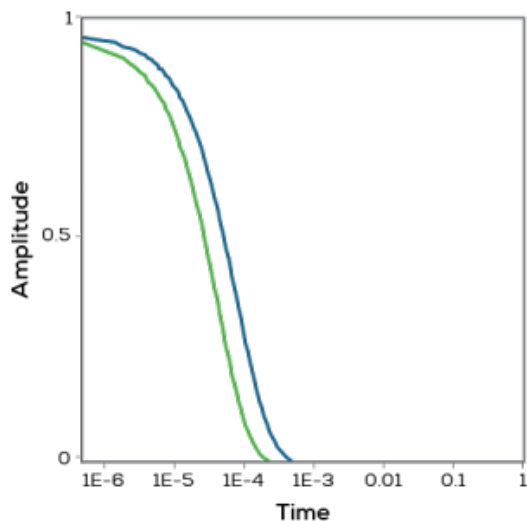

Figure 5: Correlation functions for proteins of two different sizes (green is smaller, blue is larger).

To get from a Correlation Function to the stuff you really care about – sizing data – two analysis methods are used. The first method is called the cumulants method and it applies a 'best fit' to the Correlation Function. The shape of that fit leads to a diffusion coefficient, an average size, and a size distribution. The rate of change of scattering intensity can be connected to a single diffusion coefficient – plug that into the Stokes-Einstein equation to get the average hydrodynamic diameter (Figure 6).

At a more detailed level, the diffusion coefficient is found by observing the scattering intensity I(t).

$$D_t = \frac{K_B T}{6\pi\eta D_h}$$

D_t = Diffusion coefficient (m²/s) η = Solvent viscosity (Pa.s)
K_B = Boltzmann's constant (J/K) D_h = Hydrodynamic diameter (m)
T = Absolute temperature (K)

Figure 6: Stokes-Einstein Equation.

as a function of time. This is given by the correlation function as seen in equation 2.

Equation 2
$$G(\tau) = \int \frac{I(t)\,I(t+\tau)}{[I(t)]^2}\,dt$$

The intensities are accumulated over the experiment duration and expressed as a single exponential in equation 3

Equation 3
$$G(\tau) = 1 + A\,e^{(-Dq^2\tau)}$$

where A is related to the measurement signal to noise ratio. The decay rate Dq² contains the diffusion coefficient and the scattering vector, q, as described in equation 4

Equation 4
$$q = 2\pi n\,\sin\left(\frac{\theta}{2}\right)/\lambda$$

where n is the solvent refractive index, θ is the scattering angle and λ is the laser wavelength.

The core of cumulants analysis is fitting the exponential given by equation 3 to a second order polynomial, the first term of which gives the Z-average size and the second term the polydispersity index, PdI.

The second method of DLS analysis used by Stunner is regularization analysis. That approach draws upon a library of DLS data to recreate the measured data and determine a sample's size distributions (Figure 7). Stunner with RADLS uses a proprietary version of regularization developed by Unchained Labs. Both of these analysis methods follow the latest ISO standards for particle size analysis by DLS as stated within ISO 22412:2017.
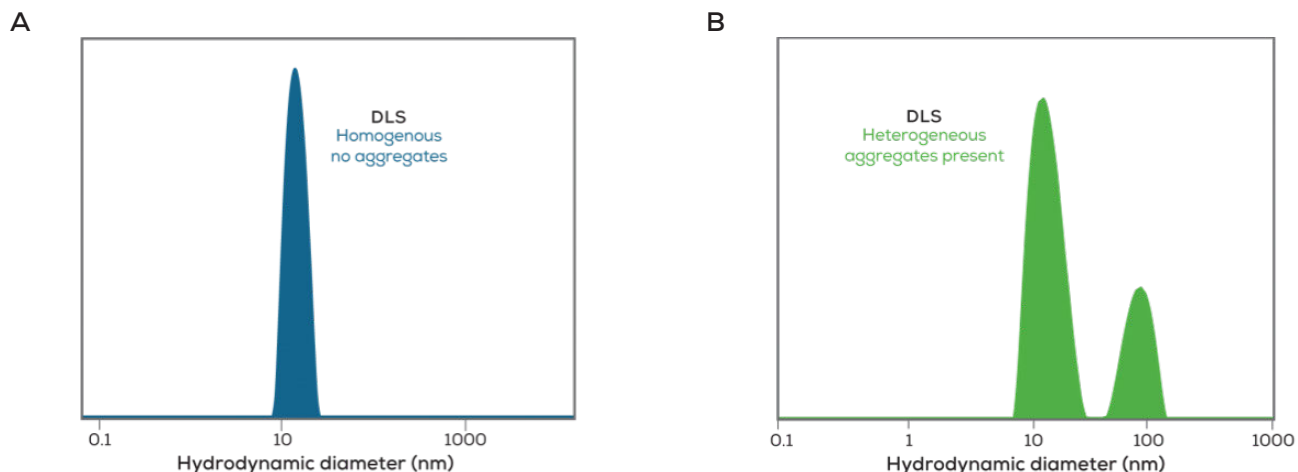
A

B

Figure 7: Size distribution of a homogenous (A) and a hetergoneous (B) sample, measured by DLS.

In the world of biologics, DLS will tell you more than the size of your sample – it is also a quick check to determine if your sample is aggregated. Since large particles scatter light more intensely, DLS can detect even very rare aggregates in a sample. Sending up a warning flag on protein aggregates can save you from relying on data from a sample already past its prime, or explain why your antibody is no longer performing at its peak.

How does Static Light Scattering work?

Static light scattering (SLS) might seem simple at first – a laser goes into a sample, hits particles, and comes back out – but static light scattering is actually one of the most powerful techniques to detect particles in solution. The intensity of scattered light is the main readout of an SLS experiment and, if you know what you expect of your sample, that intensity can tell you a lot. SLS can be used to calculate molecular weight, concentration of lipid nanoparticles, titer of viral particles, or almost anything else, and is the most sensitive way to keep an eye on when aggregation begins in a sample.

Static light scattering vs dynamic light scattering – what's the difference?

SLS is all about average intensity and can tell us about particle molecular weight and concentration in certain setups. In contrast, DLS reads how fast scattered light intensity changes over time, which provides information on diffusion rates and particle size.

You can say it's like listening to a rock concert– SLS would tell you how loud the music is and DLS would tell you what song is being played (Figure 8).

With SLS, laser light is directed at your sample and the scattered light is measured at one or more angles around the sample. The intensity of scattered light can be affected by a number of properties of your sample and setup, such as:

- Particle molecular weight
- Particle size (Rg)
- Particle concentration
- Interactive forces between particles
- Refractive indices of the particle and solvent
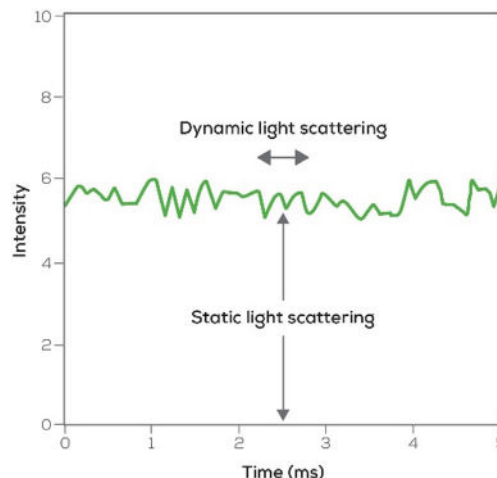- Angle between the laser and detector
- Laser wavelength

Figure 8: Schematic representation of DLS and SLS.

We can use the measured intensity to characterize the particles in your sample. As discussed above, small enough particles exhibit Rayleigh scattering equally in all directions, so measurement angle doesn't matter. Their intensity can be described by **Equation 5**:

Equation 5
$$I \propto MCn_o \left(\frac{dn}{dc}\right)^2$$

Where M is particle molecular weight, C is particle concentration, n_o is refractive index of the buffer, and dn/dc is the refractive index increment, the differential refractive index of the particle relative to its concentration. When SLS is measured it is usually reported as a Rayleigh Ratio, which is a normalized intensity of light scattering.

This kind of analysis can be used to calculate concentrations or titers from Rayleigh scattering particles – like proteins or AAV. However, in case of larger particles like LNPs or other large nanoparticles, light scattering is likely Mie scattering and an approach that looks at multiple angles is needed.

Rotating angle dynamic light scattering (RADLS)

When working with larger particles (e.g. approximately ›60 nm particles with a 660 nm laser) light scattering is angle dependent and large particles can sneak by if your detector isn't observing from a useful angle. In simple terms, imagine DLS as a way to take a 'group photo' of tiny particles using light. Just like people can appear different sizes in a photo taken from different angles, a DLS read on particle size can yield different results when measured with light at different angles. RADLS is all about taking DLS reads from multiple angles on the same sample so that you can get the best possible read on every size particle present (**Figure 9**).

RADLS is an upgrade to traditional DLS and SLS since it incorporates multiple detection angles simultaneously, providing a more comprehensive and detailed particle analysis. By collecting data at multiple angles, RADLS delivers angle independent properties such as size, polydispersity, aggregation, shape, and molecular weight of particles. This

● Larger Particle
◐ Emitter (Laser)
◐ Detector (Photon counter)
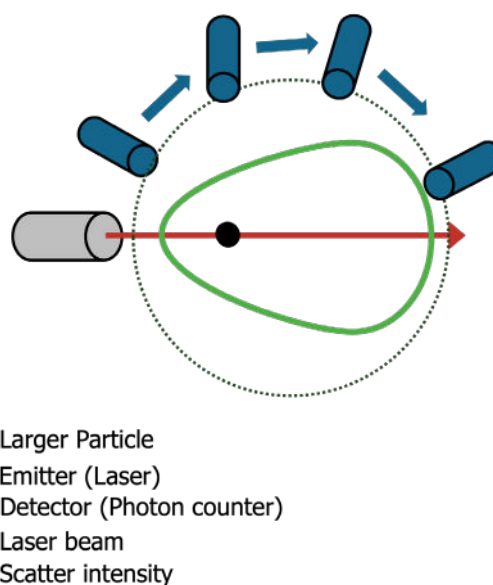—— Laser beam
—— Scatter intensity

Figure 9: Schematic representation of RADLS.

technique is particularly valuable when studying larger samples or samples with heterogeneous or polydisperse particle populations where Mie scattering causes dramatic variations in light scattering at different angles.

Stunner's RADLS technology consists of 2 lasers (each connected to an emitting fiber) and 1 receiving fiber that rotates and is connected to a photon counter. By emitting light from the top laser and rotating the receiving fiber, backward scatter angles in 110-162° range are measured. When emitting from the bottom laser and rotating the receiving fiber, forward scatter angles 30-42° can be measured.

With the ability to measure light scattering at lots of angles you could ask: what's the 'best angle' to use that can see every particle size? Theoretically, you would need to measure at a scattering angle of 0° ($\theta = 0°$) to get the actual size, but this is technically impossible since it means the detector is looking directly into the laser. The secret is to collect DLS and SLS reads at multiple angles and combine all of that data in order to extrapolate to $\theta = 0°$ mathematically.

Z-average diameter corresponds to intensity weighted average diffusion coefficient. For Rayleigh scatterers, like proteins, the intensity is independent of angle and therefore the Z-average diameter is also angle independent (**Figure 10A**), the same is true for monodisperse Mie scatterers. However, for
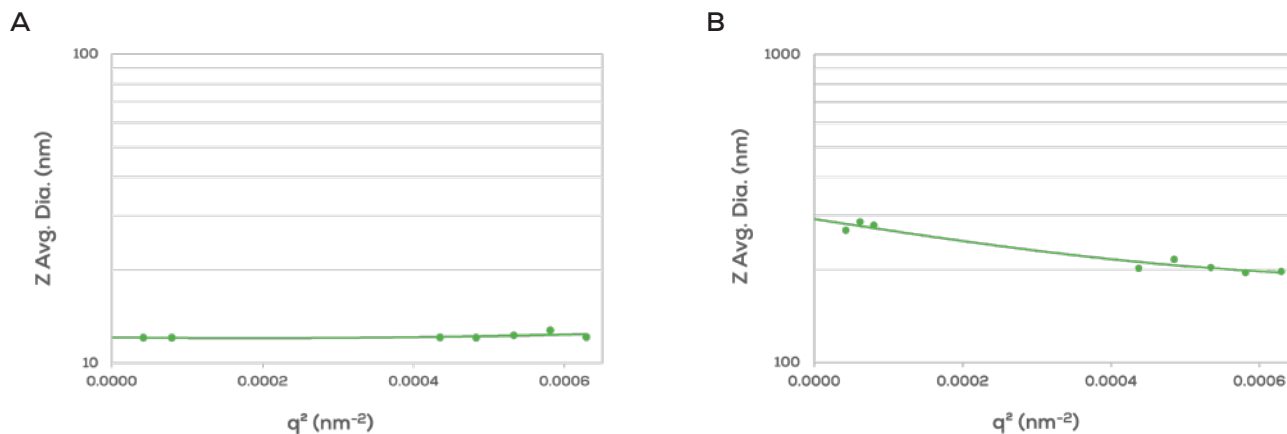
A

B

Figure 10: Z-average vs. q² for an IgG (**A**) and a LNP (**B**). Refer to equation 4 to see the relationship between angle and q².

polydisperse or multimodal samples containing a Mie scatterer, like aggregated protein or typical LNP samples, intensity will change with angle leading to a different Z-average diameter as it is intensity weighted. (**Figure 10B**). Getting Z-average diameter at each angle allows the Stunner software to extrapolate to θ = 0° and provide an angle independent Z-average hydrodynamic diameter.

And finally, let's come back to SLS where larger nanoparticles will also show anisotropic scattering effects, so if you want to measure particle concentration you need to measure intensity at multiple angles. Just like for DLS, the same logic applies that the ideal angle is 0° – but since this is not possible, an extrapolation to θ = 0° has to happen. This is what is done with a Guinier plot (**Figure 11**):
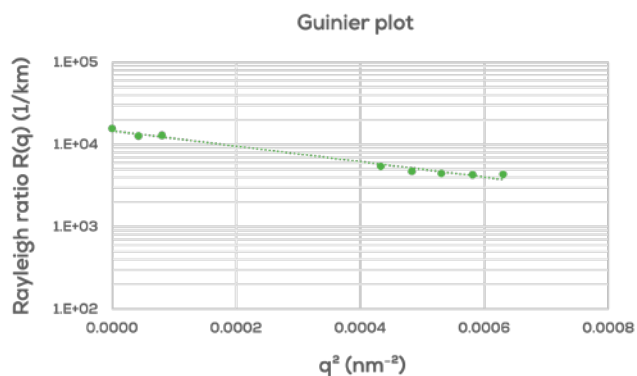
Guinier plot

Figure 11: Guinier plot of a LNP showing a change between scattering vector and light scattering as reported by Rayleigh Ratio.

This approach gives you a Rayleigh Ratio (normalized scatter intensity) at 0° and from this you can calculate particle concentration through **Equation 6**

Equation 6
$$T = \frac{R_0 N_A}{KM^2}$$

where T is the particle concentration, R_0 the Rayleigh ratio at 0°, N_A is Avogadro's number, K the optical contrast constant and M the molecular weight.

Additionally, the radius of gyration (R_g) can be retrieved from a Guinier plot. DLS leads to R_h (Hydrodynamic radius), which is your molecule's radius based on its diffusion speed and approximated by a solid sphere model. SLS leads to R_g, which is the distance from a molecule's center of mass to the point where you could concentrate all of a molecule's mass and still have the same rotational moment of inertia. What that means is your R_g will be larger for a sphere with all the mass in the outer layer and hollow in the center (like a ping pong ball) vs a solid sphere (like a golf ball) while both could have the same exact hydrodynamic radius.

R_g can be calculated from the slope of a Guinier plot through **Equation 7**:

Equation 7
$$\ln R(q) = \ln R(0°) - \frac{q^2 R_g^2}{3}$$

By combining the R_g and R_h of a sample, you can get structural information. A perfectly solid sphere (the golf ball) will have an R_g/R_h close to 0.77, while a hollow sphere (the ping pong ball) will have an R_g/R_h close to 1. Particles that are not spheres, like a nanorod, will have a larger R_g as well. By getting both R_g, R_h and their ratio as an output, Stunner's RADLS gives you additional structural insights into your sample.

Plates

Stunner measures RADLS and UV/Vis from just 2 µL of sample using the one-of-a-kind Stunner plate, which makes it possible to gather all that data from the low sample volume (Figure 12). A Stunner plate contains 96 proprietary microfluidic circuits molded from a low-absorbance cyclic olefin copolymer (COC) plastic to temporarily hold samples with no risk of cross-contamination or evaporation. Built into these circuits are the fixed pathlength microcuvettes that are used for UV/Vis and RADLS measurements, measuring 0.1 and 0.7 mm in path length (Figure 13). Both microcuvettes are used for UV/Vis reads, and the 0.7 mm cuvette is also used for all light scattering measurements.
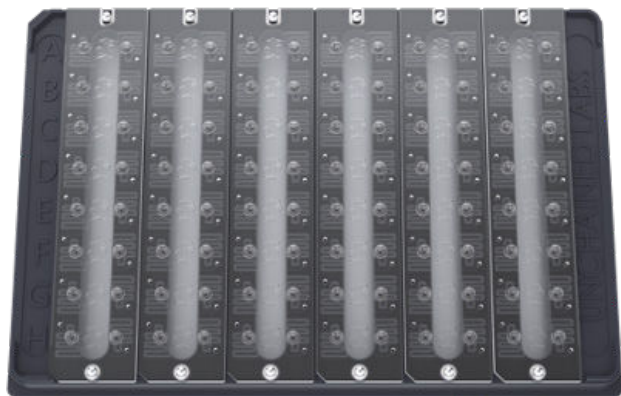

Figure 12: Strips containing 16 individual microfluidic circuits are mounted on frames to make Stunner plates.

Each circuit contains 5 main features: the input well, storage channel, microcuvette(s), overflow reservoir, and vent hole (Figure 13). Samples are deposited into the input well and drawn into the storage channel by capillary action.
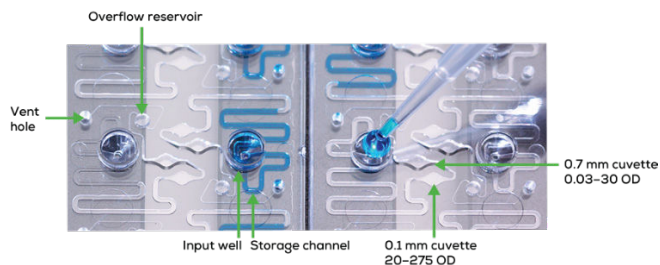

Figure 13: Close-up view of Stunner microfluidic circuits with 2 different path length microcuvettes and their dynamic ranges.

Stunner plates have the same dimensions as standard 96-well microplates. This means that Stunner can easily be integrated with liquid handlers and other lab automation to enable hands-off, high-throughput analysis.

Stunner measurements, step-by-step

Once you load a sample plate into Stunner, it completes 8 steps to make a measurement. Stunner can measure UV/Vis and RADLS on 7 angles in about 1.5 minutes per sample, or 96 samples in just over 2 hours.

1. UV/Vis: Empty beam measurement

Stunner determines the intensity spectrum of light emitted by the xenon flashlamp through air with the sample spectrophotometer and a reference detector. Stunner uses the empty beam measurements to determine the exact spectral output of the xenon flashlamp for every experiment.

2. UV/Vis: Automatic positioning

For optimal spectral measurements, Stunner positions the plate or chip so the focal point of the xenon flashlamp's light passes through the center of each microcuvette. Stunner determines the intensity of light transmitted through an empty microcuvette while making small adjustments to the plate position and repeats this process 2 more times. Based on these 3 measurements, Stunner automatically optimizes the correct plate position.

3. UV/Vis: Empty microcuvette measurement

Stunner measures the intensity spectrum of light transmitted through the empty microcuvette to remove any contribution the surrounding plastic may have on the spectrum, so you can be confident the resulting absorbances belong to the sample alone.

4. UV/Vis: Sample pumping

After measuring the empty microcuvette, Stunner connects a pump to the vent hole of the microfluidic circuit and pumps the sample through the microfluidic circuit while monitoring the change in transmitted light intensity in the microcuvette. Stunner sees when the microcuvette has been filled with sample and stops pumping, reducing measurement times.

5. UV/Vis: Filled microcuvette UV/Vis measurement

As a last step during UV/Vis measurements, Stunner measures the intensity spectrum transmitted through the filled microcuvette.

Stunner repeats steps 3–5 for each of the user specified microfluidic circuits, acquiring an empty and a full intensity spectrum. The software then uses these intensity spectra to determine the sample absorbance spectrum across the UV/Vis spectrum. All 5 measurement steps are optimized to give high quality spectral data in the shortest possible time. For more information on how Stunner uses UV/Vis spectrophotometry, check out the Tech Note "A look under the hood of Lunatic" – Stunner performs UV/Vis measurements the same way.

6. RADLS: Height sweep to find the center of the cuvette

RADLS needs to be able to measure a lot of angles without interference from the top, bottom or sides of each microcuvette, so it is important that the laser and receiver beam overlap happens as close to the middle of the microcuvette as possible. With UV/Vis, the center of the cuvette was already determined. Now the Z position needs to be set and the complete RADLS module can move up and down to achieve this. This step is repeated at different heights until the top of the cuvette is found and then an offset

is applied to get to the center. This is the best Z coordinate.

7. RADLS: Laser sweep to find the perfect beam overlap at the chosen angle

Every time the RADLS angle is changed the overlap of the emitting and receiving beam is also slightly changed – so alignment must be optimized to avoid a loss in intensity. This is what we call the self-aligning optics of the RADLS module. In step 6, the optimized height was determined but now the overlap must be optimized so that it is consistent at every angle. An X/Y sweep at the optimized height identifies the location with maximum intensity for the angle measured by RADLS.

8. RADLS: DLS and SLS measurement at various angles

The RADLS module has determined the perfect position: the center of the cuvette and the maximum beam overlap. Now it will do 5 acquisitions of 1 second each (by default, but can be set higher and longer) at this angle. Once done, the RADLS module will repeat step 7 and 8 for a single sample for 7 angles (by default, but can be set higher). After this, the module will move to the next sample and repeat steps 7 and 8 again for all angles until all samples are measured.

Conclusion

By improving on traditional DLS and UV/Vis spectrophotometry principles, Stunner with RADLS takes LNP, nanoparticle, protein and nucleic acid quantification and quality checks to the next level. The combination of innovative optical design, microfluidic-based chips with fixed pathlength cuvettes, robust signal processing, and a range of purpose-built apps means Stunner will provide state-of-the-art precise and accurate results.

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Rev A