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Bio-nano science: Better metrics would accelerate progress

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ABSTRACT An early step of evaluating a nanomaterial's potential for biological applications is investigating its interactions with cells and biological fluids. These experiments generate complex data, which are summarized and analyzed through the use of metrics. Choosing appropriate metrics is thus critical to build a foundation for nanomaterial research. Unfortunately, several widely used metrics in the bio-nano scientific literature have significant issues and may not be delivering their intended insight. This perspective will label and discuss three problematic metrics in wide use within the field: Percentage Cell Association, used to measure the interaction strength between a cell and a nanoengineered material; Internalization Factor, used to measure the ability of a cell to internalize a nanoengineered system; and Detected Protein Abundance, used to analyze the composition of proteins that adsorb to a nanoengineered surface. We review the origin of each metric and explain why each fails to deliver the intended insight in the domain of bio-nano experiments. Finally, for each metric, we present alternative metrics that deliver the necessary insight while imposing little (or no) additional experimental burden. We hope that this perspective will decrease the use of these metrics, improve reporting, and ultimately aid the transition of bio-nano science to a more quantitative field.

INTRODUCTION

Over the last two decades, nanotechnology's promise to revolutionise medicine has been widely recognised, however, realisation of this promise through clinical translation has proven to be challenging.[1–3] Key to realising the potential of nanomedicine is understanding the fundamental interactions between nanoengineered particles and cells. For example, it is well known that the physicochemical properties of particles, such as size, shape and surface charge, interact to influence interactions with cells.[4–6] However, a complete picture of this relationship has been elusive and particle design informed by the prediction of bio-nano interactions remains a distant goal.

Like much biological data, data from bio-nano experiments can be extremely complex. Central to extracting understanding from complex data is the idea of a *metric*: a quantitative measure that reduces an experimental dataset to a single number that can be reported, comprehended, and compared. Metrics are used widely in many domains, both inside and outside of science (**Figure 1**). Examples of effective metrics include: the median lethal dose (LD_{50}), which summarises how toxic a substance is; reaction rate constants, which describe reactivity between chemical species; and pharmacokinetic parameters, which summarise drug distribution and clearance from the body. When used appropriately, a metric is a powerful tool with which to reduce complexity and enable comparison. Conversely, the use of ill-defined or inappropriate metrics impedes progress, obfuscates the underlying processes, and may lead to unproductive investigation, limiting our understanding.

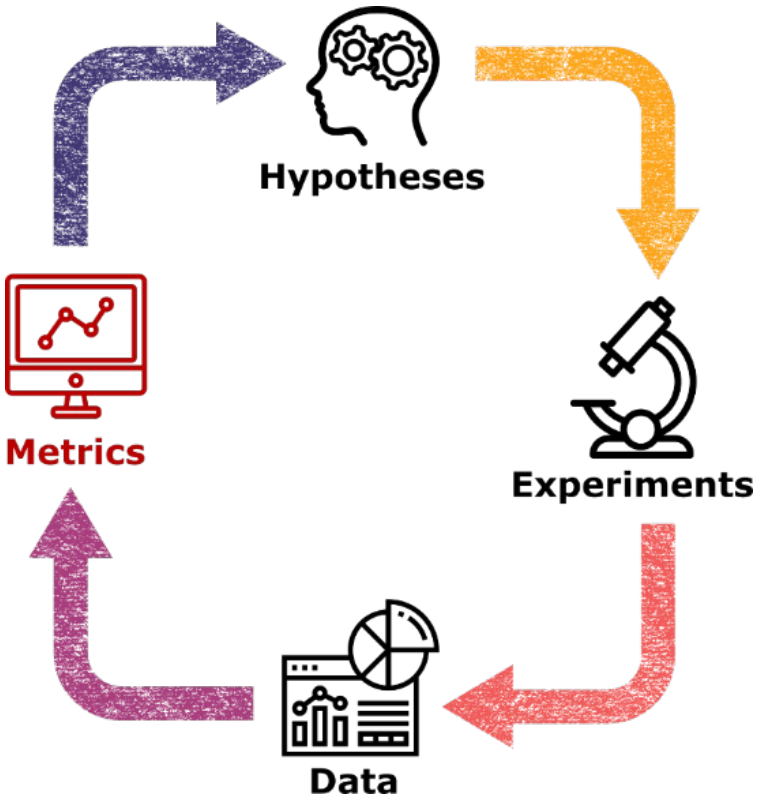

Figure 1. The role of metrics in the scientific process. Hypotheses drive experiments. Experiments produce data. Data is analysed and summarised using metrics, which generate new hypotheses. Flawed metrics impede scientific progress.

The multidisciplinary nature of bio-nano science means that many of the metrics in use have been adopted from other fields. Unfortunately, when applied to problems in the bio-nano domain, several widely used metrics fail to provide the insight they deliver in other contexts. In this focus article, we label and discuss three metrics that fall into this category: **Percentage Cell Association** (%CA), which is used to measure the interaction strength for a particular particle-cell pairing; **Internalisation Factor** (IF), which is used to quantify the ability of a specific cell type to internalise a particular type of particle; and **Detected Protein Abundance** (DPA), which is used to analyse the composition of the proteins that adsorb on the surface of a particle (the

biomolecular/protein corona). We use the term *particle* here loosely to refer to a wide variety of nanoengineered systems.

We will define each metric, including the context in which it was developed and its flaws when applied to bio-nano data. Additionally, we suggest alternatives for each metric that can better capture the desired insight. These alternatives require minimal additional experimental effort, and instead focus on changes to the analysis of the experimental data. By improving the metrics we use, we can move bio-nano science from qualitative, 'one-off' investigations into a more quantitative field.

PERCENTAGE CELL ASSOCIATION (%CA)

%CA is a popular metric for analysing flow cytometry data of cells that have been incubated with particles.[7,8] This can be expressed as either a ratio or a percentage and is also sometimes referred to as Percentage (Ratio) of Positive Cells or Percent (Ratio) Uptake. %CA is defined as *the percentage of cells that have a detectable number of particles associated to them*. Basic flow cytometry assays do not provide subcellular localisation information, so 'associated' particles may have been internalised or may be bound to the cell membrane (with sufficient strength that the washing steps of an experimental protocol do not remove them). %CA is expressed as:

$$\%\mathrm{CA} = \frac{\mathrm{C_{assoc}}}{\mathrm{C_{assoc}} + \mathrm{C_{none}}} \times 100 \qquad (1)$$

where C_{assoc} is the number of cells with a non-zero (detectable) number of associated particles and C_{none} is the number of cells with zero (detectable) associated particles. To calculate %CA, the signal intensity is measured for each cell within a population, typically using a fluorescent flow cytometer. This cytometry data is subsequently gated into two populations: one

representing cells with associated particles (C_{assoc}) and the other representing cells without associated particles (C_{none}). The number of cells in each population is used to calculate %CA.

%CA has its origins in immunophenotyping analysis of flow cytometry data, in which it is often desirable to both identify cell subpopulations and determine how common they are relative to other cells.[9] This is typically accomplished by labelling a complex mixture of cells with a variety of different immunolabels; analysis of this multiparametric flow cytometry data is achieved through Boolean gating and determination if cells are present (or absent) within these gates.[10] In this context, where staining for a label is typically bimodal and discrete, the percentage of cells positive for a label or labels (analogous to %CA and sometimes called *percent of parent*) is a quantitative and valuable reporting metric, as the central question is *whether* a cell subpopulation contains the labelled features. The actual amount of feature that was labelled is a secondary concern.

Unfortunately, the utility of %CA is substantially diminished when assessing the *strength* of the interaction between a particle system and a cell line, for the following reasons: First, it does not distinguish variation in the number of associated particles. Each cell is counted as either associated or non-associated. Thus, a cell that is associated with a thousand particles contributes the same amount to %CA as a cell that is associated with only a single particle (assuming detection is sufficiently sensitive to detect a single particle over cellular background). Large variation in association can occur, both when comparing different particle-cell experiments and within a single experiment. We argue that a particle system in which *more* particles associate to a cell type interacts more with that cell type, and a metric of association should reflect this.

Second, %CA has a built-in bias towards particle systems with a higher labelling intensity. (**Figure 2(a)**). The *brighter* a particle is, the more it shifts the intensity for an associated cell,

increasing the chance that associated cells will fall within the *associated* gate. Of course, this apparent higher association for brighter particles is an artefact.

Third, this metric is highly sensitive to experimental and analytical variability. A small change in the positioning or size of the gates can significantly alter the resulting curve (**Figure 2(b**)). Gates are typically subjectively and manually determined for each experiment (as opposed to being generated algorithmically). A small subpopulation of non-associating cells will also have a strong effect on %CA.

Fourth, as a population level metric (a population of cells results in a single number for %CA), statistics and distributions are challenging to obtain and require a far greater number of experiments.

Ultimately, although %CA can provide a basic, qualitative test of whether cells and particles associate at all, it is unsuitable for quantitative comparison. Median fluorescence intensity (MFI) of the sample is a more informative measure which solves many of the issues with %CA. However, MFI is also unsuitable for comparison between different experiments and particle systems as it depends on instrumentation settings and also favours brightly labelled particle systems.

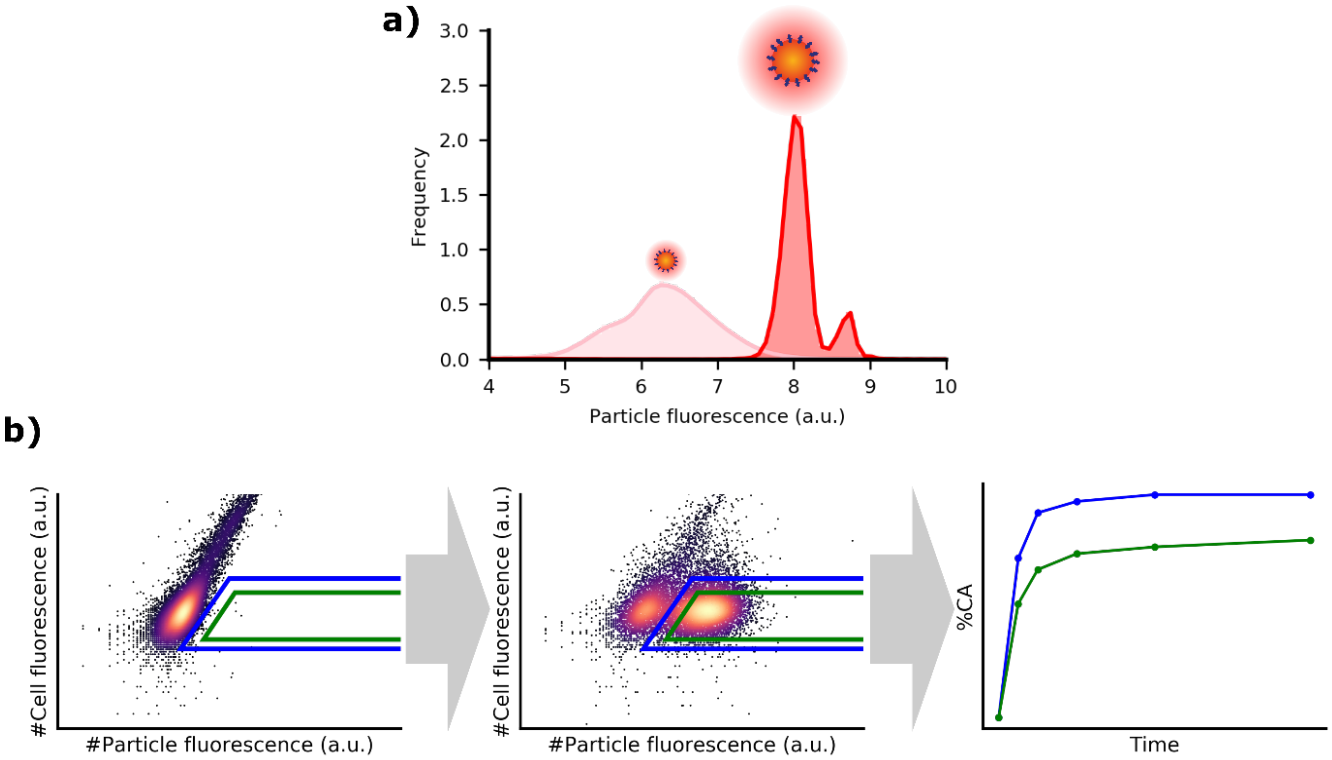

Figure 2. Illustration of some issues with the %CA metric. (a) Brighter-labelled (e.g., larger) particles have an inherent advantage. (b) Gates are typically drawn manually and can greatly alter the resulting %CA curve.

An improvement to MFI is to remove the background fluorescence of cells and divide by the fluorescence per particle to obtain an estimate of the number of particles associated per cell:

$$\text{particles per cell} = \frac{\left(F_{exp} - F_{cell}\right)}{F_{particle}} \quad (2)$$

where F_{exp} is the MFI of the experimental sample, F_{cell} is the fluorescence intensity of a cell (i.e., autofluorescence or a cell-only control sample), and $F_{particle}$ is the fluorescence intensity of a particle (i.e., a particle-only control sample). Equation (2) provides an estimate of the number of particles associated per cell, an instrumentation-independent measure of cell-particle association. Similar techniques have been used to quantify particle uptake since at least the 1980s.[11–14] This

approach does have caveats in that both particle and cell fluorescence may have a wide distribution which depends on cell type.[15] Additionally, some fluorescent labels will change their intensity based on environment (for example, when internalised by cells), and thus photostable labels are recommended when quantifying particle-cell interactions in this manner. Alternatively, corrections can be made for changes in fluorescence due to the environment.[13] We note that this metric is not restricted to fluorescently labelled particles and can be just as easily applied to mass cytometry data with metal-labelled particles.

Even estimating *particles per cell* does not take into account variability in the dose of particles delivered to the cell surface, which can vary considerably in different investigations.[16,17] This *delivered dose* is affected both by experimental parameters (e.g., particle concentration) and by particle physicochemical properties (e.g., density, size) which lead to variability in settling behaviour. To directly compare experiments performed with different systems or under different conditions, it is necessary to isolate the biological kinetics from the experimental conditions. We have recently introduced a method that achieves this,[14] which can be easily applied to new data using an online tool at **http://bionano.xyz/estimator.** The output of this analysis is the *affinity* between a cell line and a particle system, analogous to a rate constant in chemical reactions.

INTERNALISATION FACTOR (IF)

IF (also called Internalisation Feature and Internalisation Score) is a widely used metric for quantifying cell-particle internalisation. In the simplest terms, this metric is the ratio of total fluorescence intensity located inside the cell to the total fluorescence intensity in the image. In bio-nano experiments, this is equivalent to the ratio of the number of particles internalised by a cell to the number of particles associated with that cell (where associated includes both internalised and bound to the cell membrane). IF is calculated[18] as:

$$\mathrm{IF} = \log\left(\frac{\mathrm{a}}{1-\mathrm{a}}\right) \tag{3}$$

$$\mathrm{a} = \frac{\mathrm{m_I}}{\mathrm{m_I} + \mathrm{m_B}}\left(\frac{\mathrm{p_I}}{\mathrm{p_B}}\right) \tag{4}$$

where m_I, m_B are the mean intensity of the upper quartile of pixels inside the cell and in the membrane of the cell, respectively; and p_I, p_B are the peak intensity of the upper quartile of pixels inside the cell and in the membrane of the cell, respectively. Determining these variables relies on defining image masks that delineate which part of a microscopy image is the interior and/or membrane of a cell. Unlike %CA, each cell in a population has an IF, and these are typically averaged over a large population for reporting. Calculating and reporting IF has become routine for researchers who use imaging flow cytometry, as it is implemented in the software for the most popular commercially available imaging cytometers.[19]

This metric does not appear to have been reviewed or discussed in peer-reviewed literature. Thus, tracing the history of this metric is challenging, though we suggest it may have been inspired by approaches that determine protein localization within cells, where the ratio of a specific quantity of protein inside the nucleus is compared to the quantity in the entire cell.[20]

A metric based on ratio of fluorescence intensities (whether IF or something simpler) is a practical, quantitative measure in specific biological contexts. For example, when investigating where a fluorescently labelled protein is located or expressed inside a cell, IF provides a value that increases when there is more protein inside the cell and decreases when there is more protein in the cell membrane.[21] An implicit assumption in this analysis is that an experiment is merely *labelling* features that are already present. Unfortunately, when used to quantify the degree or likelihood to which cells internalise a labelled material that has been *externally introduced,* such as a particle, IF is misleading and impedes understanding.

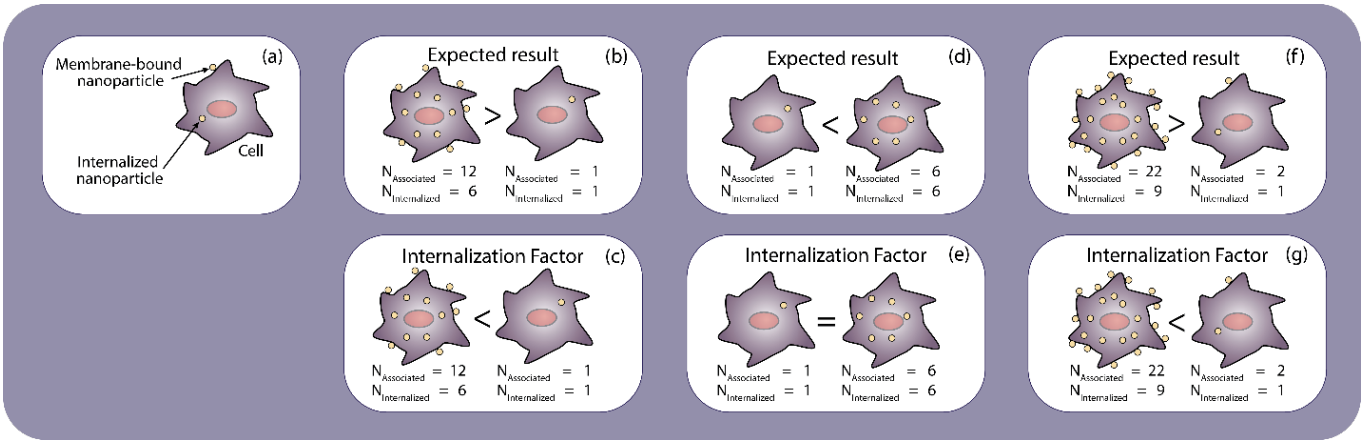

Figure 3. Issues with the Internalization Factor metric. (a) Schematic of membrane-bound and internalised particles. (b, d, f) Comparison of the internalisation ability of cells using an intuitive metric. (c, e, g) Comparison of the internalisation ability of cells.

These issues with IF are most easily conveyed visually. Consider the two cells in **Figure 3(b,c)**. Certainly the cell on the left has internalised more particles than the cell on the right (6 vs 1), and we might expect an internalisation metric to assign a higher value to the left cell than the right. Unfortunately, IF assigns a higher value to the cell with one internalised particle and no membrane-bound particles (**Figure 3(c)**). **Figure 3** contains additional examples where IF provides values that contrast with our expectation.

The number of particles inside a particular cell depends not only on how well a cell internalises particles, but also on a number of upstream processes: how many particles the cell has been exposed to, how well particles adhere to the cell membrane, and how well particles are transported into the cell. Downstream processes within the cell (e.g., breakdown or exocytosis of particles) will also affect the number of internalised particles. It may be tempting to think of IF as isolating the process of particle internalisation from earlier cellular processes. However, this interpretation is incorrect: each imaging flow image is a snapshot of a cell in time, and we do not

know *when* a specific particle was encountered. A particular particle could be on the outside of a cell because the rate of internalisation was slow or because the cell encountered the particle later in the incubation. Additionally, this snapshot does not account for how many particles a cell was exposed to that *did not* bind (or were weakly bound and removed with washing steps). It is not currently possible to determine how many particles a specific cell encountered (or when a particle was encountered) merely from imaging flow data. Thus, a different metric must be used if we seek to understand and represent particle internalisation.

Fortunately, like %CA, most of the issues associated with IF can be easily rectified without performing additional experimental work. To calculate IF, both the fluorescence intensity internal to the cell (defined by an image mask) and the membrane or external intensity must be known. This internal intensity is far more indicative of cell-particle internalisation than IF. A step beyond that is to normalise internal intensity to the average intensity of a single particle; as with %CA this provides an estimate of total particles internalised per cell (equation (1)). Particles internalised per cell is an absolute measure, independent of instrumentation settings, and is thus more valuable for comparing experiments. Determining internalisation using microscopy in this manner has been used in other contexts.[6,13] Like %CA, considerations of the dose of delivered particles is also essential for absolute quantitation of cell experiments. As above, this requires the use of computational modelling tools to provide kinetic estimates. A separate issue for this technique is the information loss when converting from a 3D object (cell with particles) to a 2D image. It is not straightforward to differentiate between an internalised particle, and a surface membrane-bound particle in the centre of the cell image. This imposes limits to the accuracy of 2D microscopy methods to assess internalisation; however, even with these restrictions we believe imaging flow cytometry is a valuable tool.

DETECTED PROTEIN ABUNDANCE (DPA)

A major area in bio-nano research is how the physicochemical properties of a particle influence biomolecular corona formation.[22,23] The pristine surface of nanomaterials is fouled due to the presence of biomolecules present in serum and plasma, which adsorb onto the particle. Understanding the composition of the biomolecular corona is critical, as cells will first interact with the corona rather than the surface of the particle. Particle association and uptake are thought to be dominated by these cell-biomolecule interactions rather than direct cell-particle interactions.

Characterisation of the biomolecular corona consists of identification and quantification of proteins present. While simultaneous identification and quantification is not always performed, it can be considered the gold standard of corona characterisation and is typically evaluated using liquid chromatography coupled with tandem mass spectrometry. The output of these identification and quantification assays can take several forms, notably normalised spectral abundance factor[24] or relative abundance by mass.[25] For brevity, we refer to this family of metrics as DPA, though we note that interpreting raw proteomic data to identify and quantify the proteins present is a complex problem with competing approaches.[26]

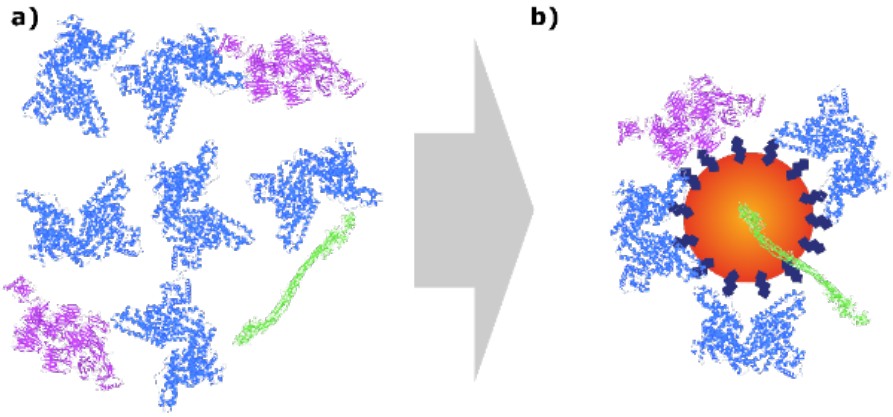

Figure 4. Issue with the DPA metric. (a) High abundance of a protein (blue, albumin) in a biological fluid is likely to lead to (b) high abundance of the protein in a sample when a particle is exposed to the biological fluid.

Typically, in bio-nano science, a DPA metric will be reported for each protein identified in the corona, and this will be considered a full characterisation. The issue with this metric is that not all proteins are equally abundant in biological fluids. Consider if, after immersion of a particle in a biofluid, we find that the DPA of protein A is 70% and that of protein B is 30%. Naively, protein A has a higher affinity to the particle. However, if we consider the biofluid to consist of 99:1 A:B then clearly, protein A is underrepresented in the corona while protein B is substantially overrepresented, suggesting that protein B has a higher affinity. Given that 'the goal of the identification process is generally to identify as many proteins as possible',[26] we should expect the identification of highly abundant proteins regardless of their affinity for a particular particle (**Figure 4**).

In proteomics, DPA is an early step in an analysis pipeline that incorporates background levels of protein expression. Unfortunately, measurement and analysis that include background levels of proteins are rarely reported in bio-nano science. Instead of DPA, we recommend also characterising proteins or other biomolecules present in the biofluid, and performing a differential expression analysis for species in the corona:

$$P_{i,expression} = \frac{P_{i,sample}}{P_{i,background}} \quad (5)$$

where $P_{i,sample}$ is the DPA of protein *i* in the sample and $P_{i,background}$ is the DPA of protein *i* in the background. Differential expression analysis is a standard technique in proteomics and has been used previously for the analysis of biomolecular coronas on both inorganic[27,28] and organic[24] particles. There are of course challenges here – notably the large dynamic range of protein concentration within some biological experiments. We also note the importance and difficulty of properly reporting proteomics experiments[29] and welcome bio-nano standardisation in this area.[30]

CONCLUSIONS

Metrics can provide simple, quantitative summaries of complex data, aiding comparison and communication. Comparing two values of the same metric obtained from two experiments should immediately suggest which experiment is closer to achieving its goal, be that particle uptake, development of non-fouling systems, or demonstrating biocompatibility. We have highlighted the limitations associated with three widely used metrics, and the pitfalls that arise from their use.

We suggest that current limitations in understanding various processes in bio-nano science are at least partially driven by the choices made when selecting a metric to summarise experimental

data. An 'ideal' metric should account for variation in the experimental conditions, such that metrics obtained from different experimental configurations can be meaningfully compared. Thus, metrics in use may change as our understanding develops. In part, this motivated the MIRIBEL (Minimum Information Reporting in Bio–Nano Experimental Literature) guidelines,[31] which encourage the reporting of experimental conditions to account for apparent differences in reported metrics.

The metrics discussed here all have significant flaws and we strongly discourage their use. Fortunately, many of these flaws can be addressed without significant changes in experimental practice. Indeed, in certain cases, all the information required to improve the metric is already contained within the data obtained while performing the experiment. For both %CA and IF, we recommend at least estimating the number of particles associated with each cell, see equation (2). For DPA, we recommend at least a differential expression analysis, see equation (5).

These changes in reporting and analysis are not onerous, yet the benefits can be immense. The reporting of robust metrics allows for meaningful quantitative analysis to be performed, adding value to experimental data aside from the conclusions immediately drawn from that data.

Choosing appropriate metrics is often *necessary* for making sense of complex data, but it is not *sufficient*. Appropriate statistical analysis is also vital; but a "one-size-fits-all approach to statistical inference is an inappropriate expectation[32]". Still, two general principles are worth bearing in mind. First, distributions and uncertainty estimations are far more valuable than merely stating an average or a summary statistic. Second, biological replicates are typically far more valuable than technical replicates.[33] These principles are especially relevant to flow cytometry based methods, where only averages of technical replicates are sometimes reported; despite the high-dimensional and rich statistical information the technique can provide.

Statistical analysis beyond labelling something as "significant" is an important and coming change to current scientific practice, and we refer readers to a recent issue on this topic.[32] For additional information on planning cytometry experiments, we refer to a recent correspondence[34].

Nanoengineered systems still retain an undeniable potential for changing the medical landscape. However, we cannot hope to fully unlock this potential without understanding the fundamental biology, chemistry and physics that govern bio-nano interactions. Robust metrics provide a stepping-stone: an opportunity to bridge the gap in our collective understanding and lay a concrete foundation upon which progress can be built.

AUTHOR CONTRIBUTIONS

M.F. and S.T.J. performed formal analysis, investigation, visualization, and produced the initial draft. All authors were responsible for reviewing and editing, conceptualization, and methodology. E.C., F.C., and M.F. performed supervision and project administration.

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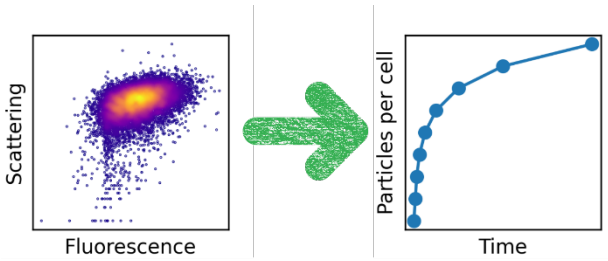
Scattering
Fluorescence
Particles per cell
Time
Metrics simplify complex data

TOC Graphic

Biographical Sketches

Matt Faria currently holds the Rejane Langlois Research Fellowship in the Department of Biomedical Engineering at the University of Melbourne. Prior to this, he received his PhD in Biomedical and Chemical Engineering and conducted postdoctoral research as part of the ARC Centre of Excellence in Convergent Bio-Nano Science and Technology. His research interests include quantitative biology, rational pre-clinical evaluation of nano-therapeutics, tighter integration between biological experimentation and computational modelling, and synthetic biology.

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