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Particle size analysis — Particle tracking analysis (PTA) method

Analyse granulométrique — Méthode d'analyse de suivi de particule (PTA)

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Foreword

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The committee responsible for this document is Technical Committee ISO/TC 24, *Particle characterization including sieving,* Subcommittee SC 4, *Particle characterization*.

Introduction

Regulatory, scientific and commercial requirements for nanomaterial characterization or characterization of particulate suspensions where particle sizing and counting is required provide a strong case for further development of techniques such as Particle Tracking Analysis (PTA), also known as Nanoparticle Tracking Analysis (NTA) [14]. Due to the fact that the term PTA covers a larger size range and is more generic[1)], the term PTA is used throughout this document to refer to NTA and PTA. For all aims and purposes, the term PTA also means NTA in this document.

PTA is based on measuring the diffusion movement of particles in a suspension by means of laser illumination, imaging of scattered light, particle identification and localization, and individual particle tracking[2)]. In this case, suspension is an even dispersion of particles, gas bubbles or other liquid droplets. The hydrodynamic diameter of the individual particles, droplets or bubbles is related to Brownian motion parameters via the Stokes–Einstein equation.

In recent years the academic community working in fields such as liposomes and other drug delivery vehicles, nanotoxicology, viruses, exosomes, protein aggregation, inkjet inks, pigment particles, cosmetics, foodstuffs, fuel additives and fine bubbles began using the PTA technology for characterization. An ASTM standard guide (E2834–12) [10] was developed to give guidance to the measurement of particle size distribution by means of Nanoparticle Tracking Analysis. The present document aims to broaden the scope of the specification and to introduce system tests for PTA operation.

This document outlines the theory and basic principles of the particle tracking analysis method along with its limitations and advantages. It also describes commonly used instrument configurations and measurement procedures as well as system qualifications and data reporting. One of the key aspects is the meaning of the data and its interpretation. It should be noted that the key measurand obtained from PTA measurement is the number-based particle size distribution where the size is taken to mean the hydrodynamic diameter (3.11) of the particles in the sample. This size can be different from other sizes obtained with different techniques such as dynamic light scattering [6] or electron microscopy [4].

1) NTA is the most recognised abbreviation for the technique described in this document. However the Particle Tracking Analysis (PTA) includes NTA in its size range of measurements.

2) For the purpose of this document “tracking” will mean “following in terms of particle x and y position” and the “track” will mean “the path of that particle defined by such x and y coordinates of each step”

Particle size analysis — Particle tracking analysis (PTA) method

1 Scope

This document describes the evaluation of the number–based particle size distribution in liquid dispersions (solid, liquid or gaseous particles suspended in liquids) using the particle tracking analysis method for diffusion velocity measurements.

2 Normative references

There are no normative references in this document.

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at http://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
nanoscale
length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE: ISO/TS 80004-1:2015, 2.1]

3.2
nano-object
material with one, two or three external dimensions in the *nanoscale* (3.1)

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1:2015, 2.5]

3.3
nanoparticle
nano-object (3.2) with all three external dimensions in the *nanoscale* (3.1)

Note 1 to entry: If the lengths of the longest to the shortest axes of the nano-object differ significantly (typically by more than three times), the terms *nanofibre* or *nanoplate* are intended to be used instead of the term nanoparticle.

[SOURCE: ISO/TS 80004-4:2011, 2.4]

3.4
particle
minute piece of matter with defined physical boundaries

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects* (3.2).

[SOURCE: ISO/TS 80004-6:2013, 2.9]

3.5
agglomerate
collection of weakly bound particles or *aggregates* or mixtures of the two where the resulting external surface area is similar to the sum of the surface areas of the individual components

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces, or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-4:2011, 2.8]

3.6
aggregate
particle comprising strongly bonded or fused particles where the resulting external surface area may be significantly smaller than the sum of calculated surface areas of the individual components

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent bonds, or those resulting from sintering or complex physical entanglement.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO/TS 80004-4:2011, 2.7]

3.7
particle size
linear dimension of a *particle* (3.4) determined by a specified measurement method and under specified measurement conditions

Note 1 to entry: Different methods of analysis are based on the measurement of different physical properties. Independent of the particle property actually measured, the particle size can be reported as a linear dimension, e.g. as an equivalent spherical diameter.

[SOURCE: ISO/TS 80004-6:2013, 3.1.1]

3.8
particle size distribution
distribution of *particles* (3.4) as a function of *particle size* (3.7)

Note 1 to entry: Particle size distribution may be expressed as cumulative distribution or a distribution density (distribution of the fraction of material in a size class, divided by the width of that class).

[SOURCE: ISO/TS 80004-6:2013, 3.1.2]

3.9
equivalent diameter
diameter of a sphere that produces a response by a given particle-sizing method, that is equivalent to the response produced by the particle being measured

Note 1 to entry: The physical property to which the equivalent diameter refers is indicated using a suitable subscript [ISO 9276-1:1998].

Note 2 to entry: For discrete-particle-counting, light-scattering instruments, an equivalent optical diameter is used.

Note 3 to entry: Other material constants like density of the particle are used for the calculation of the equivalent diameter like Stokes diameter or sedimentation equivalent diameter. The material constants, used for the calculation, should be reported additionally.

Note 4 to entry: For inertial instruments, the aerodynamic diameter is used. Aerodynamic diameter is the diameter of a sphere of density 1 000 kg m^{-3} that has the same settling velocity as the irregular particle.

[SOURCE: ISO/TS 80004-6:2013, 3.1.5]

3.10
light scattering
change in propagation of light at the interface of two media having different optical properties

[SOURCE: ISO 13320:2009, 3.1.17]

3.11
hydrodynamic diameter
equivalent spherical diameter of a particle in a liquid having the same diffusion coefficient as the real particle in that liquid

[SOURCE: ISO/TS 80004-6:2013, 3.2.6]

3.12
particle tracking analysis
PTA
method where particles undergoing Brownian motion in a liquid suspension are illuminated by a laser and the change in position of individual particles is used to determine particle size

Note 1 to entry: Analysis of the time-dependent particle position yields translational diffusion coefficient and hence the particle size as hydrodynamic diameter using the Stokes-Einstein relationship.

Note 2 to entry: Nanoparticle Tracking Analysis (NTA) is often used to describe PTA. NTA is a subset of PTA since PTA covers larger range of particle sizes than *nanoscale* (3.1).

[SOURCE: ISO/TS 80004-6:2013, 3.2.8, modified — Nanoparticle tracking analysis has been removed from the term, and Notes 1 and 2 have been modified.]

3.13
nanomaterial
material with any external dimension in the *nanoscale* (3.1) or having internal structure or surface structure in the nanoscale

[SOURCE: ISO/TS 80004-1:2015, 2.4]

3.14
diluent
non-volatile homogeneous liquid which is used to decrease the number concentration of *particles* (3.4) in a suspension without any deleterious effects such as changing particle total number, state of aggregation, *particle size* (3.7) or surface chemistry

3.15
viscosity
measure of the resistance to flow or deformation of a liquid

[SOURCE: ISO 3104:1994]

3.16
percentile
value of a variable below which a certain percentage of observations fall

[SOURCE: ISO 11064-4:2013, 3.7]

4 Symbols and abbreviated terms

For the purposes of this document, the following symbols and abbreviated terms apply.

CCD	Charge Coupled Device		
CMOS	Complementary Metal Oxide Semiconductor		
CV	Coefficient of Variation (standard deviation divided by arithmetic average)(ISO 27448:2009, 3.11)		
CCD	Charge Coupled Device		
d	hydrodynamic diameter	metre	m
D_x	translational diffusion coefficient in 1 dimension		m^2/s
D_{xy}	translational diffusion coefficient in 2 dimensions		m^2/s
D_{xyz}	translational diffusion coefficient in 3 dimensions		m^2/s
η	viscosity of the suspension medium	pascal second	Pa·s
k_B	Boltzmann's constant		$m^2\ kg\ s^{-2}\ K^{-1}$
RSD	Relative Standard Deviation (ISO/TR 13843:2000, 2.34)		%
T	absolute temperature	kelvin	°K
t	time	second	s
$\overline{(x)^2}$	mean square displacement in 1 dimension	metre squared	m^2
$\overline{(x,y)^2}$	mean square displacement in 2 dimensions	metre squared	m^2
$\overline{(x,y,z)^2}$	mean square displacement in 3 dimensions	metre squared	m^2

5 Principles

5.1 General

Determination of particle size distribution by PTA makes use of the Brownian motion and light scattering properties of particles suspended in liquids. Irradiation of the sample (typically by means of a laser beam of wavelength in the visible region) leads to light scattering by objects with a refractive index that is different from that of the surrounding medium. Light scattered from each particle is collected by magnifying optics and visualized by way of a suitable detector, such as a Charge Coupled Device (CCD) or Complementary Metal Oxide Semiconductor (CMOS) camera. By recording a series of sequential images, the instrument's software tracks positions of particles as a function of time, allowing analysis of their movement.

By tracking individual particles, undergoing random Brownian motion [6] [13], from frame[3] to frame, the average spatial displacement of the particles per unit time can be calculated, and this displacement

3) For the purpose of this document, "frame" will mean a still image obtained from video capturing of the moving objects in PTA measurement equipment".

can be related to the hydrodynamic diameter of the particles through the Stokes-Einstein equation[13]. Although translational Brownian motion is a three-dimensional process, it is possible to use a one-, two-, or three-dimensional diffusion coefficient to determine particle hydrodynamic diameter. The relevant formulae are derived in Annex A and can be summarized with three formulae below:

$$\overline{\left(x\right)^2} = D_x t = \frac{2k_B Tt}{3\pi\eta d} \tag{1}$$

$$\overline{\left(x, y\right)^2} = D_{xy} t = \frac{4k_B Tt}{3\pi\eta d} \tag{2}$$

$$\overline{\left(x, y, z\right)^2} = D_{xyz} t = \frac{2k_B Tt}{\pi\eta d} \tag{3}$$

Mean square displacement $\overline{\left(x\right)^2}$ can be measured in x and y directions independently to give two independent values for particle size [Formula (1)]. In most PTA instruments, $\overline{\left(x, y\right)^2}$ is evaluated as shown in Formula (2). It should be noted that in all three cases there is no assumption of two dimensional movement of particles. All particles are assumed to be moving freely in all three dimensions while the measurement is sampling the projection of each x, y and z component of that movement onto the xy observation plane. As described in Annex A, these components (observables) are independent variables

5.2 Key physical parameters

Formulae (1) to (3) show that as well as the diffusion coefficient, the temperature and the viscosity of the sample shall be known in order to calculate the hydrodynamic diameter.

5.3 Detection limits

Like any measurement technique, PTA has detection limits in terms of the particle size and the particle number concentration. These limits are heavily dependent on the particle material, diluent and polydispersity of the sample.

Depending on the physical properties of the particles, the typical working range of the PTA can be from about 10 nm to about 2 µm in diameter.

5.3.1 Lower size limit

The lower limit of detection in terms of the particle hydrodynamic diameter is determined (apart from sensitivity and dynamic range of the camera) by the light scattering from the particles. It is the combination of refractive indexes of the particle material and the diluent that affect the amount of light scattering the detection and tracking system. A large difference in refractive indexes results in higher scattering and therefore lower detection limit for all other parameters being the same.

Better tracking of highly scattering particles results in preferential counting of particles. The accuracy of counting is covered in 5.4.4.

Sample polydispersity affects the ability to track and therefore analyse different size fractions in the particle number-size distribution. The underlying effect is linked to the dynamic range of the video capture and image analysis. In a polydisperse sample large particles scatter a lot more than small particles making it difficult to detect or track small size particles. All the values in Table 1 are given for monodisperse samples. In the case of a monodisperse gold spheres in suspension, the lower limit of detection is typically 15 nm but can range from approximately 10 nm to 20 nm.

Below is the table of detection limits for commonly used dispersions (particle-diluent combinations).

Table 1 — Lower limit of detection for monodisperse suspensions of nanoparticles

Particle material	Approximate lower detection limit (Hydrodynamic diameter in nm)
Gold	15
Polystyrene	45
Silica	75
Biological materials	60
Other metals or metal oxides	25

General effects of samples and measurement parameters on the detection limits are described in the subclauses below. The typical values quoted for room temperature water dispersion are provided in Table 1. These values are approximate and could vary (as much as 30 %, for example leading to low detection limit for gold ranging from approximately 10 nm to 20 nm) depending on factors such as porosity of silica or the type of biological material.

5.3.2 Upper size limit

The upper particle size limit is limited by slowing Brownian motion at larger particle sizes. The motion of such particles is very slow and long observation periods may be required. Very large particles can also produce so much scattering that the detection system may not track much smaller particles in the same polydisperse sample.

In the limit of very large particles (or gas bubbles) the sample may separate with heavy particles sedimenting (or large bubble creaming). These effects shall be considered at all times for PTA measurement.

5.3.3 Sample and sampling volume

In a number of applications the knowledge of sample volume and sampling volume involved in a PTA measurement can be important. Typically used equipment requires approximately 1 ml of sample to be used for measurement.

The subsampling methods can vary between manufacturers, yet the sampling volume of liquid that is being investigated within the PTA microscope field of view[4)] is often limited to a range of approximately 0,1 nl to 1 nl volume. The sampling volume, for the PTA measurement is limited laterally by the optical field of view of the system to (typically of the order of) 100 µm by 100 µm area. The particles in that area are tracked using imaging power of the optics with an approximate focus depth of (the order of) 10 µm which is taken as the sampling volume depth. This results in a sampling volume of 0,1 nl. Larger sampling volumes may be obtained for optical systems with larger field of view or lower magnification.

In order to obtain a representative measurement of a sample (especially for low particle number concentrations), the sampling volume should be increased. This is often achieved by sampling multiple parts of the sample and performing a new measurement as described in Clause 7.

5.3.4 Maximum particle number concentration

When preparing samples or evaluating the applicability of PTA to existing samples, the limits of particle number concentration shall be considered. This subclause addresses the limitations of the PTA method in terms of the particle number concentration. All references in this document to concentration are made to particle number concentration and not molar concentration or mass concentration due to the nature of the measurement. Appropriate conversions can be made from (for example) mass concentration to particle number concentrations at sample preparation stages. PTA requires highly diluted samples and the optimal particle concentration is sample-dependent. The optimal particle concentration should be

4) As defined in ISO 10360-7:2011, 3.3, “field of view” is the area viewed by the imaging probing system.

assessed by preliminary tests on a series of dilutions. Depending on the level of prior knowledge about the sample concentration, selecting an appropriate dilution can take several iterations.

The number of particles in the sampling volume of the instrument determines the number of tracks and therefore the quality of the statistical result of the measurement. The more particles tracked, the more representative the obtained particle distribution is. However, too large a number of particles affects the ability to independently track particles in the field of view (see 5.3.3). If particle paths intersect (which happens in highly concentrated samples), the tracks are rejected leading to less tracked data.

A typical value of maximum particle number concentration measurable with PTA is 10^9 particles per ml.

5.3.5 Minimum particle number concentration

Every PTA measurement shall contain enough particle tracks to reach a specified level of sampling repeatability. In principle it is possible to track just one particle, but this may not be representative or reproducible enough for a required measurement. So the minimum particle number concentration is determined by the sampling level of the result the user is trying to achieve (see 5.4). In addition, if the user is evaluating the particle size distribution over a wide range of sizes, the minimum particle number concentration required is larger as there are more size bins[5)] over which the tracking data should be spread.

For instrument systems with a wide optical field of view/sampling volume (larger than that in 5.3.3), the minimum measureable particle number concentration can be as low as 10^6 particle per ml.

5.4 Measurement precision and uncertainties

5.4.1 General

It is important to note that although PTA usually involves the simultaneous tracking and analysis of multiple light-scattering particles, the diffusion coefficient and hence the hydrodynamic diameter of each particle is determined individually before the data are integrated to produce a number-based particle size distribution. It should be noted that the data processing algorithms can vary between manufacturers.

5.4.2 Measurement precision

The number of tracks of different particles determines the level of representative sampling. Tracking enough particles for an appropriate statistical representation of the sample is important [2][4].

An example of an approximate relationship between the number of tracks, number of frames, video length and the PTA measurement precision expressed in CV of modal particle size for a monodisperse 100 nm sample is reported in Table 2.

Table 2 — Approximate values showing dependence of PTA measurement on the number of tracked particles, number of frames, video length

Number of tracks	Number of frames	Video length s	CV of modal particle size %
400	130	5	< 10 %
700	230	8	< 8 %
1 000	300	10	< 5 %
2 000	600	20	< 3 %
NOTE Data were obtained for a 100 nm monodisperse sample of polysterene spheres.			

5) For the purpose of this document, “size bin” refers to the particle diameter size range width which corresponds to a given (size-number) histogram particle number count.

Table 2 should be used as a guidance in planning experiments.

For all the data sets in Table 2, the size bins for the particle size distribution were kept at 5 nm. Other size bins may be used in other measurements. Larger size bins allow better count statistics but compromise size resolution.

For a given bin size, the number of tracks recorded can be used as an indication of the data precision. For such a Poisson distribution, the square root of the number of tracks in a size bin represents the precision estimate. An assessment of precision should be implemented by sampling three or more times the same sample and a CV be obtained for each size bin.

The precision of PTA measurement is affected by a number of factors including polydispersity, distribution of particle track lengths and size. Thus the values in Table 2 represent a relatively ideal case. This means that, to achieve a desired precision, a higher number of tracks should be counted for more polydisperse samples. Methodology described in ISO 13322-1:2014, Annex A[4] ab-initio calculation of the number of tracks required for a given precision. Table 2 indicates the minimum number of frames or video length to achieve a given CV of modal particle size. Number of tracks, number of frames and video length follow an approximate linear relationship (within the experimental fluctuations of Table 2 data).

5.4.3 Size range

Particle tracking size limits have already been described in 5.3.1 and 5.3.2 regarding the lower and upper size limits. It was also mentioned that the particle size range can affect the measurement accuracy due to the effect of larger particles on counting smaller ones in a polydisperse sample.

A larger particle size range also affects the number of tracks allocated to each size bin in the particle size distribution. Increasing the size range without increasing the number of size bins results in a coarser size granularity in the particle size distribution. The user of this document should be guided by the accuracy required for each size bin and ensure enough tracks are sampled in each size bin. This means that the total number of tracks required for a larger size range can be notably larger.

5.4.4 Counting efficiency

Most PTA systems give an indication of the number of particles in the field of view and this can be used to estimate the total number concentration in the sampling volume (see 5.3.3). The uncertainties involved in this calculation are related to the optical properties of the instrument and the polydispersity of the sample. Due to a finite depth of focus (typically ~10 μm), the particles are tracked and counted in that volume only.

Measurement of particles critically depends on the ability of the PTA system to detect them. Larger particles can be detected with more ease than smaller ones. Samples that contain particles of very different sizes may therefore oversample (or overcount) larger particles. Due to the statistical nature of this measurement the particle track lengths vary. Tracks that are too short or intersecting tracks are rejected by the processing software. Some instrument manufacturers employ an optimization procedure that optimises the threshold automatically whereas some allow users to set the minimum threshold of tracks manually.

Another effect of large particles is related to their dynamics, smaller particles on average move greater distances than larger particles between frames. In some cases, these particles exit the field of view and in that case may be disregarded from the calculation. Larger particles at the same edge of the field of view are more likely to be tracked for longer thus contributing more significantly to the overall count. Conversely, a small particle outside the field of view has a greater chance of more rapidly entering the field of view than a large particle at that same position. Therefore, providing the number of particles of a given size *per frame* are being reported on, this in itself does not lead to a bias in the measurement.

5.4.5 Sizing accuracy

Based on Formulae (1) to (3) in Clause 5, the particle diameter (hydrodynamic diameter, d) depends on uncertainty in the temperature of the sample, in the tabulated value of viscosity (which is itself temperature-dependent) and on the error involved in measuring the particle mean displacement while tracking.

An uncertainty of ± 3 K in temperature results in approximately ± 1 % additional error in diameter evaluation. The temperature of the sample shall therefore be measured to better or equal to ± 3 K for this standard to be applicable. It is also required to bring the sample and instrument into thermal equilibrium before starting any measurement. A freshly injected sample should be allowed to equilibrate for 1 min to 2 min before measurement.

Values for viscosity will often be taken from tabulated values for a given diluent. Viscosity varies with temperature. Such variation shall be less than ± 2 % over the operational range of temperatures used in an experiment. In the case of water at approximately room temperature, the ± 2 % requirement for the accuracy of the viscosity implies a more stringent requirement on knowledge of the absolute temperature (than ± 3 K) as the tabulated viscosity data and temperature are interdependent.

Each particle track contains a number of steps over which the particle has been tracked. The longer the particle is tracked, the greater the accuracy of the evaluation of its hydrodynamic diameter (providing zero bias) [12]. The uncertainty in evaluating the $\overline{\left(x\right)^2}$ [see Formula (1)] for each particle is therefore proportional to $1/\sqrt{n_{\text{steps}}}$, where n_{steps} is the number of steps in each track.

Any vibration or movement that is not a result of particle Brownian motion will lead to a decrease in the reported size of particles, and as such shall be avoided. Therefore, steps shall be taken to prevent vibration affecting the measurement. The tracking software shall be capable of detecting non-Brownian motion and either correcting for it or at least alerting the operator to its presence.

5.4.6 Size resolution

Size resolution refers to the ability to distinguish two closely size-related particle populations. This parameter is determined by the uncertainty in measurement accuracy of a single particle, the reproducibility of the data in each size bin as well as the counting efficiency (see 5.4.4) in each size class of the particle distribution.

6 Apparatus

PTA equipment will generally comprise a common collection of basic components, with the possibility of additional peripherals that may be desirable or required for specific experiment types. The components of the core apparatus are described below.

Figure 1 illustrates a common geometry of the PTA experimental setup. It should be noted that the orientation shown implies the particle tracking in the *xy* plane. The arrangement of optical illumination and detection may also be rotated about *x* axis with the CCD Camera pointing along the y direction. The right angles between the illumination laser and the optical detection is not a requirement as other angles allowing dark field imaging of the sample are commonly used.

See Annex B for information on apparatus settings and best practice.

6.1 Sample cell (with sample in dispersion). The sample to be analysed is held in the sample cell. This cell shall be inert to the sample, and shall be able to hold the sample at a stable thermal equilibrium. The temperature of the sample and cell shall be measureable, and as a minimum requirement the temperature of the sample shall be measured with ± 3 K precision. The cell shall, at least in part, be optically transparent to allow illumination of the sample by the radiation source and collection of scattered light by the optical assembly.

6.2 Laser (or another light source). The laser source shall be such that the intensity and wavelength provide appropriate scattering from particles without bleaching, destroying or modifying them in any way. The wavelength and intensity shall also be appropriate for image collection by the digital camera. The beam shall be focused to maximize illumination of in-focus particles, and to minimize optical noise generated by the illumination of out-of-focus particles. The irradiation shall give rise to minimal localized heating or photophoresis.

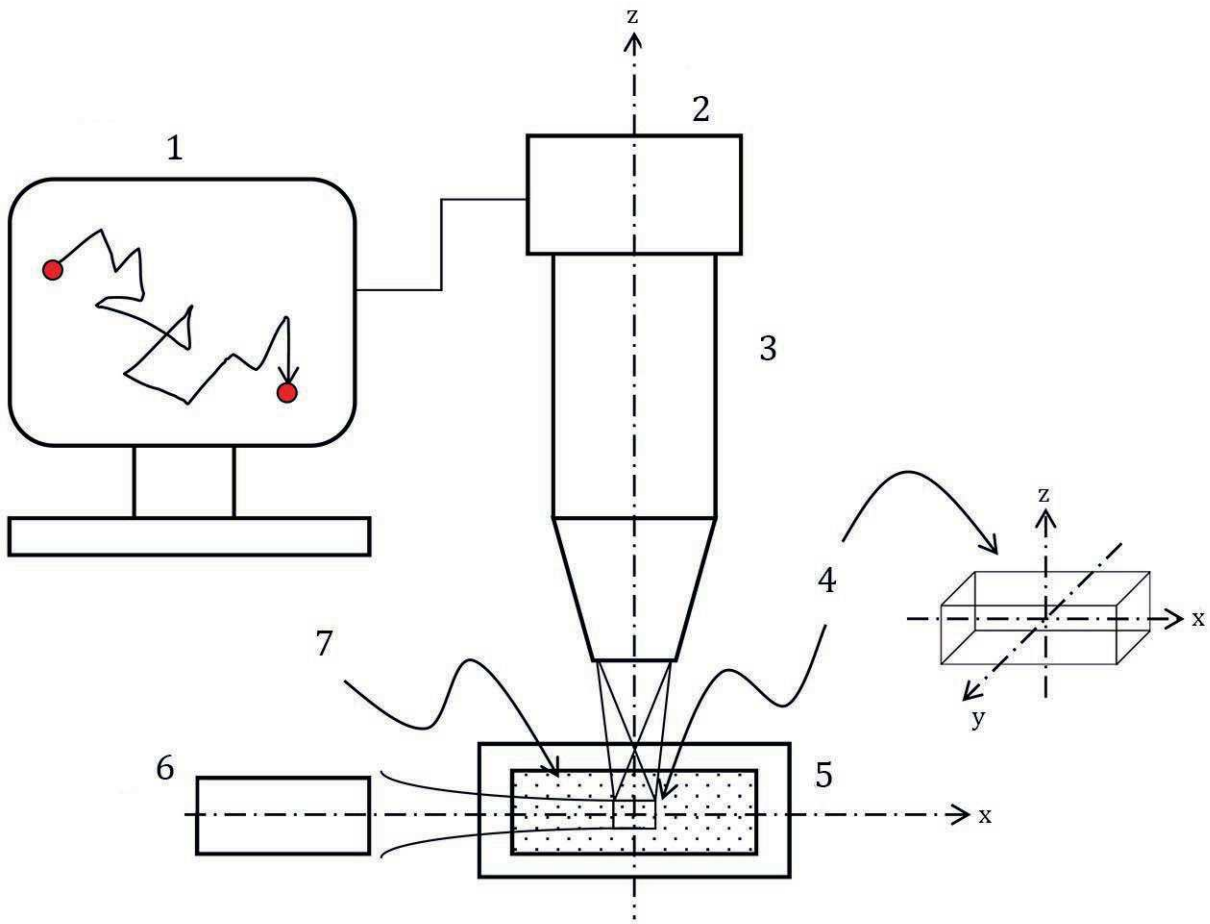
For certain experimental procedures, such as the visualization of fluorescently labelled particles against a non-fluorescent scattering background, the radiation shall be monochromatic and the wavelength matched to both the excitation wavelength of the fluorophore and the optical filters used in signal collection.

6.3 Optical microscope (with optical magnification). Scattered (or emitted) light is collected and delivered to the image capture apparatus through a series of lenses, filters and mirrors generally resembling a conventional optical microscope. It is worth noting that magnification sufficient to image the particles is unnecessary, the only requirement being sufficient resolution to allow suitable measurement of the motion of the particles being measured.

6.4 Digital video camera. The image capture apparatus is a digital camera equipped with either a CCD or CMOS detector sensitive enough to image the light scattered by the particles in the sample. The camera frame rate (typically 10 to 60 frames per second) shall be such that sufficient displacement data are collected for each particle, allowing accurate particle tracking and determination of particle size. It should be noted that the camera frame rate is not typically adjustable by the user and instead is more commonly set by the choice of camera by the manufacturer.

6.5 Tracking and data processing computer. A video of illuminated particles undergoing Brownian motion shall be captured. The video shall be analysed using particle tracking software. The software should be capable of processing each of the video images; identifying, localizing, and counting individual particles; tracking discrete particles from frame to frame by recognizing the same particle in separate

frames of the video; calculating the diffusion coefficient of each particle; and integrating and outputting the gathered data.



Key

1	tracking and data processing computer	5	sample
2	sensitive digital camera	6	laser
3	optical magnification	7	sample in dispersion
4	sampling volume		

Figure 1 — Schematic representation of the PTA experimental setup

7 Procedure

7.1 General

This clause describes procedural steps for the PTA measurement. Some manufacturers may have specific recommendations for each stage but the schematic below aims to outline common process flow.

The schematic in Figure 2 describes the process flow of the PTA measurement. Solid lines indicate compulsory steps in the process while dotted lines indicate optional steps and procedures. Optional steps help improve data noise levels and repeatability.

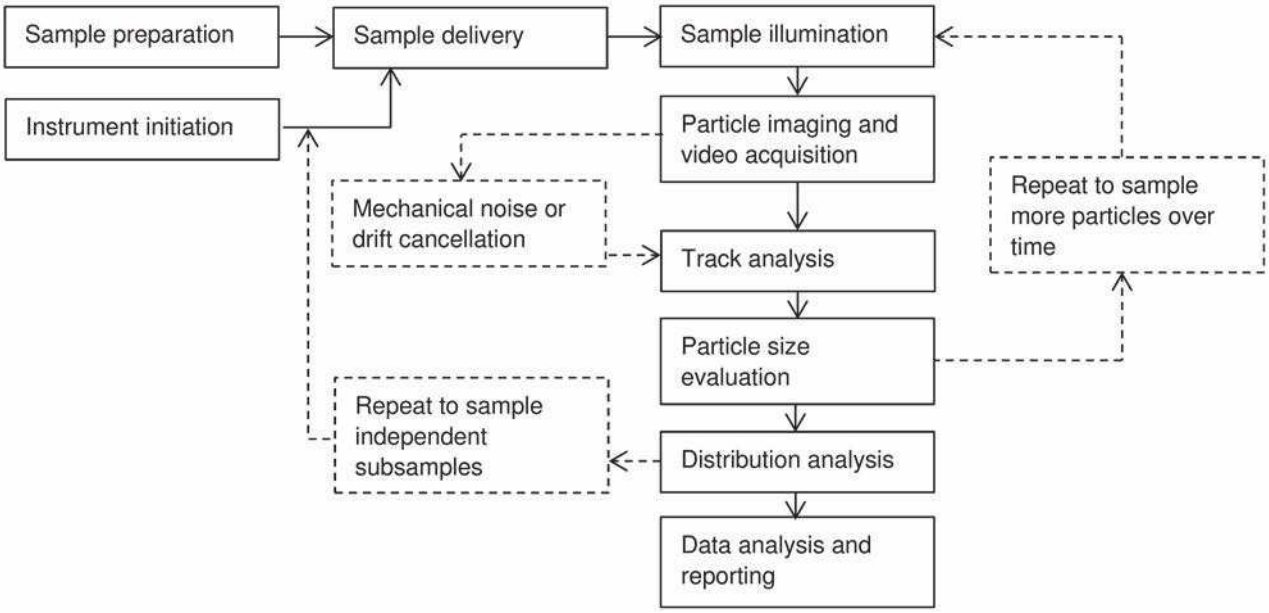


Figure 2 — Schematic of PTA measurement procedure

7.2 Sample preparation

The determination of particle size distribution by PTA requires small volumes of sample (see 5.3.3) dispersed in a suitable liquid. It is important to ensure that the sample analysed by the PTA instrument is representative of the population being investigated. ISO 14488 includes guidance on the problems of representative sampling. In the case of powders which shall be dispersed before analysis, consultation of ISO 14887 is recommended. Appropriate sample preparation and sampling are critical to reproducible measurements.

Samples shall be homogeneously dispersed in a suitable liquid (diluent) such that particles and diluent have different refractive indices. The diluent will be a transparent medium[6]. The diluent shall be compatible with both the test sample, i.e. it shall not affect the physicochemical properties of the dispersed particles and PTA apparatus. The diluent shall form a stable suspension of particles, and be of a suitable viscosity at the measurement temperature to allow a useful magnitude of particle displacement via Brownian motion. The value of viscosity should be known within ± 2 % over the operational range of temperature to be used.

The user shall ensure that the sample holder is free of contamination or other particles that can contribute to the measurement. The cleaning shall be performed according to manufacturer's specifications.

Prior to dispersion of the sample it shall be ensured that the diluent is free of interfering particles. If the dilution of the sample shall be performed, a compatible particle-free diluent shall be used, with the same refractive index, concentration of salts, surfactants, pH, etc., as the original medium in order not to change the surface chemistry of the particles. Dilution of the sample shall be such that particles can be clearly individually localized and tracked, while allowing sufficient particles to be tracked. Typically measurable particle number concentration can vary from 10^6 to 10^9 particles per ml (see 5.3.4 and 5.3.5).

7.3 Instrument set-up and initialisation

Before measurements of particle size distribution are performed, it is necessary to initialise the instrument. Power shall be supplied to software, image capture equipment, irradiation source, temperature control and readout, and any motors, valves, or pumps. Correct communication between

6) A transparent medium is a medium in which the transmission is not significantly attenuated (ISO 22891:2013, 3.9).

the various components shall be verified, and the system shall be allowed to reach thermal equilibrium in order to provide the most stable environment possible. The sample temperature shall be known to better than ± 3 K and be stable over the measurement period.

The first settings to be optimised should be the coordinates of particle observation. Adjust the field of observation in the x, y direction by movement of the optics or of the sample chamber and in the z-direction by adjustment of the focus. The observed position shall be such that illumination of visible particles is maximised, background interference is minimised, and as large a proportion of particles as possible can be seen in sharp focus.

Once the correct region of the sample is in focus, suitable image capture settings should be selected. These can include camera gain and shutter, gamma correction, greyscale conversion and analysis duration. Settings shall be appropriate for a particular sample under investigation. For example, small, weakly scattering particles will demand relatively long shutter times and high gain settings to allow sufficient signal to be detected for tracking purposes, while large, strongly scattering particles require the opposite to minimize saturation of the image. A fast frame rate (10 to 60 frames per second) is appropriate for small, rapidly diffusing particles, but is limited by the requirement of a suitable exposure time in each image. The analysis duration necessary is a function of the degree of repeatability required, with increased analysis duration leading to an increasingly representative sampling of the particle population (see 5.4.2).

7.4 Measurement

7.4.1 Sample delivery

This sample may be introduced manually or automatically (with optional motorised dilution and delivery). The sample should remain stable in the sampling volume (see 5.3.3) and shall avoid significant particle drift or particle immobilisation (e.g. sedimentation in case of large particles or creaming in case of bubbles).

Most suppliers provide a way to compensate for a small constant drift velocity making its effect on the measurement negligible. However, a fast particle drift can limit the time for particle tracking and can therefore affect the measurement statistics.

7.4.2 Sample illumination

The sample is illuminated by a laser source (6.2) or another strongly directional light source) with a wavelength that is appropriate for an optimum contrast of the sample (medium and particles). Typical wavelengths can range from 400 nm (violet) to 650 nm (red).

The sample should be illuminated to give the best contrast for particle tracking. This means that particles should be clearly visible on the video capture system (6.5) while the background level should be minimised. Poor contrast in images can result in lower number of smaller particles being successfully tracked. This, in turn, can therefore affect the resulting particle size distribution by reducing the number of smaller particles.

Note that the image size calibration (nm/pixel) is usually performed by the manufacturer and should not require the attention of the operator. However, if the instrument has been modified or new optics have been installed then such a calibration should be performed according to the manufacturer's instructions.

The contrast of the imaging system shall be optimised by focusing the illuminating laser on the sample volume and by also focusing the imaging lens system for maximum particle clarity. Some parameters are adjusted automatically by the equipment. These procedures may vary for different manufacturers. The user of this document shall follow the manufacturer's recommended settings and procedures to improve the imaging contrast.

7.4.3 Particle imaging and tracking

After thermal stabilization and drift settling of the sample and the selection of suitable method parameter settings, one or more videos of the illuminated sample may be recorded. Typically if recording more than one video, then the sample should be changed (new sample should be delivered to the measurement cell) to introduce an entirely new particle population. In line with other particle size analysis methods, it is necessary to have at least 3 such independent sub-samples. The number of aliquots that need to be analysed under repeatability conditions depend on the target measurement uncertainty to be achieved. The length of the video and number of tracks required was discussed in 5.4.

7.4.4 Track analysis

After selection of the analysis settings, the centre of each particle in every frame of the video is located by the software, and for each particle the distance moved between every frame of video in which it is visible is measured. Over the course of a single video, hundreds or thousands of particles can be identified and tracked, and the mean squared displacement between frames is calculated for each particle individually. This mean squared displacement per time is used in conjunction with the known temperature of the sample and the viscosity to calculate the hydrodynamic diameter of the observed particles on a particle-by-particle basis. Finally, particle size data are combined to produce a particle size distribution in either histogram or frequency per size bin format.

Visual inspection of the particle tracks on the screen can give a good estimate for the data quality obtained from the particle tracks. A constant drift, mechanical noise patterns (all tracks have similar shapes) or a one off (non-statistical jump) behaviour of a single particle can reduce the ability to track or even measure the particle size from such a data. A visual inspection of tracks can help the user to establish the level of confidence in the sample under test.

7.5 Results evaluation

7.5.1 General

The algorithms for data analysis may vary between manufacturers. This subclause deals with common steps in data analysis and interpretation of the results.

7.5.2 Particle size evaluation

Particle size is evaluated according to the derivation in Annex A and summarized in Formulae (1) to (3) in Clause 5. It should be noted that the size of the particle refers to the hydrodynamic diameter (see 3.11).

7.5.3 Distribution analysis

The results from each track are analysed and placed in the size bins to construct a particle size distribution. In order to achieve a required uncertainty, a certain minimum number of particle tracks should be used to fill the size-bins in the particle size distribution as described in 5.4. Data quality control shall therefore be in place to ensure that enough data points populate the size bins. A very noisy histogram usually means that not enough tracks were measured. Rerunning the tracking process on the same sample more than 3 times gives a repeatability evaluation for each size bin calculated as standard error of the mean for each size bin.

7.5.4 Data analysis and reporting

Data reporting may vary between manufacturers; however, the key parameters needed for a complete data report on the PTA measurement should follow Clause 10.

8 System qualification and quality control

8.1 General

The PTA system shall comply with the basic requirements outlined in this clause. These requirements are placed on system installation, maintenance, operation and qualification. These requirements should be considered as minimum requirements since some manufacturers can impose their own system-specific maintenance requirements.

Periodic verification of instrument performance is necessary for quality control purposes, and should normally be carried out by the analysis of particle size standards with metrologically traceable certified values.

8.2 System installation requirements

Installation requirements for the PTA system are commonly specified by the manufacturer. The manufacturer guidelines may include a more detailed list of technical steps and checks before the system is used. The list below represents a minimum requirement for the operation of the PTA system.

The equipment shall be located in the area free from direct sun light and away from strong heat sources (such as radiators). The area should be well ventilated and void of excess humidity (humidity should be less than approximately 45 % ± 10 % RH) and dust. Equipment storage requirements shall be taken from the manufacturer's manual.

The equipment may be installed on a vibration isolation table. This is not a strict requirement as small vibration noise can be compensated by PTA tracking software. However, if such vibration compensation is not available, it can contribute to apparent larger particle movement and therefore systematically smaller sizes of particles detected. In this case, vibration isolation shall be implemented.

8.3 System maintenance

All required maintenance procedures in the manufacturer's guide shall be followed. Below are some general maintenance steps that are common to all PTA systems.

The PTA system should always be left clean and dry whenever the system is not in use. After each use, the system shall be flushed with diluent liquid to remove all traces of sample from the tubing and optical surfaces. If using diluent with a high concentration of dissolved solids (i.e. saline solution), flush clean water through the system after use. The controlling software should be shut down.

Optical components of the system (laser, lenses, etc.) shall be kept clear of the solvent or other contaminants at all times.

8.4 System operation

Before the operation of the PTA system, it is important to undertake some control steps to ensure there is no cross-contamination with previous runs and that the system is functioning within specifications. Below are the general steps appropriate to PTA equipment operation.

— Check that the system is clean and that the buffer or dispersing liquid used does not contain contaminant particles.

— Check that the optical part of the system is optimised (clear sharp image of particles and an appropriate illumination level) as described in 7.4.2.

— The image capture should be optimised to capture the maximum number of particles by adjusting the grey scale histogram for the image capture (see 7.4.2). Camera shutter and camera gain can be adjusted to obtain this result. For the fluorescence type of experiment an appropriate filter should be used. Triggered laser pulsing may be used to reduce the effect of photo-bleaching.

— If the illumination source is temperature controlled, then appropriate thermal equilibrium should be reached before the measurements. Also check that the sample temperature is settled (±3 K with minimal overshoot). Typical settling times may vary, but could be approximately a few minutes. Checking for external noise contributions may not be necessary as most commercial systems have good mechanical and acoustic noise compensation routines.

8.5 System qualification

The most appropriate system qualification is the ability of the PTA instrument to measure the known number-based particle size distribution of a reference material correctly. Ideally, this reference material should be of similar material, particle size and particle concentration to the real sample.

PTA results are method-defined; hence comparison of measurement results with certified values only makes sense if the certified values were also derived by PTA. If such CRMs are not available, values derived by DLS for highly monodisperse suspensions of spherical particles can be an alternative. Alternatively, results from other methods obtained on spherical particles can be used.

Most reference materials are monodispersed while real samples are often not. Ideally, one uses a CRM consisting of polydisperse particles of the same material, certified for the number distribution obtained by PTA. The nearest objective test of the system can be performed on a certified reference material such as a 100 nm and 150 nm sized spherical particle sample. Sensitivity and measurement confidence of the system should therefore be assessed independently using such reference materials. Resulting system confidence should be reported with the results of the real sample measurement.

Certified reference materials with narrow size distribution for one or several of their modes such as 5 % CV (equal to standard deviation divided by average diameter of the mode) with average particle diameter (as measured by another standardised technique such as DLS) of different sizes (e.g. 100 nm and 150 nm) shall be used. For such dispersions, the modal particle size as measured by PTA shall be within ± 6 % of the stated mean size. See Table 3.

Table 3 — Accuracy requirements summary

	Deviation from the mean certified values	CV of the measured modal values	Recommended number of analysed tracks
100 nm sized particle	±6 nm	±6 %	1 000
150 nm sized particle	±9 nm	±6 %	1 000

It is also recommended that in addition to the two tests (100 nm and 150 nm sized particles) above an internal reference sample is developed that is similar to the sample in shape, size and material type to the samples to be investigated.

DLS is used as a comparative technique as it can give the closest reference to the hydrodynamic diameter of the reference material. Scanning electron microscopy or other similar techniques cannot be appropriate in this case.

The number of tracked particles has a direct influence on the statistics of the PTA measurement. The number concentration of reference material samples shall be matched where possible to the real sample and be guided by 7.2 of this document.

In a PTA measurement, a number of particles are tracked to obtain a statistical ensemble of the sample. It is understood that tracking more particles leads to an increased confidence in the measurement. An exact calculation of the number of particles needed for a given level of confidence can be found in ISO 13322-1:2014.

9 Data recording

This clause deals with the data that needs to be recorded by the instrument software in order to evaluate data and report it in the format prescribed in Clause 10. It should be noted that there can be some variation on the internal data handling, but for maximum traceability the equipment should record the calculated size of each particle successfully tracked, along with the length of time (in terms of number of frames) for which it was tracked.

The following experimental conditions should be recorded:

a) PTA equipment model reference or serial number;

b) camera type;

c) camera shutter time (ms);

d) camera gain;

e) number of frames per second, (fps);

f) temperature (K) ;

g) viscosity (mPa·s) (3.15);

h) total frames;

i) processed frames;

j) valid tracks;

k) overall drift (nm/s);

l) *X*-Drift (nm/s);

m) *Y*-Drift (nm/s);

n) manufacturers settings for camera (nm/pixel);

o) measurement uncertainty level — this parameter is obtained independently from measuring a reference sample as described in 8.4.

For maximum reproducibility of the results, all data required for the test report (Clause 10) shall be recorded. That record shall include some of the data records already outlined in this clause.

10 Test report

In addition to the requirements laid down in ISO/IEC 17025[5] ,the test report shall contain at least the following information:

a) a title (e.g. “Test Report”);

b) the name and address of the laboratory, and the location where the tests were carried out, if different from the address of the laboratory;

c) PTA equipment model reference or serial number and software version;

d) the name and address of the customer;

e) unique identification of the test report (such as the serial number), and on each page an identification in order to ensure that the page is recognized as a part of the test report, and a clear identification of the end of the test report;

f) a description of, the condition of, and unambiguous identification of the item(s) tested;

g) reference to the sampling plan and procedures used by the laboratory or other bodies where these are relevant to the validity or application of the results;

h) modal particle size;

i) $X_{10,1}$ (nm) - percentiles (see 3.16) of 10 % of cumulative distribution by number;

j) $X_{50,1}$ (nm) - percentiles (see 3.16) of 50 % of cumulative distribution by number;

k) $X_{90,1}$ (nm) - percentiles (see 3.16) of 90 % of cumulative distribution by number;

l) average particle size, x_{PTA};

m) the number of individual measurements upon which reported average values are based;

n) all the information required for the complete identification of the sample, including details of particle shape and homogeneity (if known);

o) the suspension conditions;

   1) diluent liquid and its filtering procedure (if applicable),

   2) number concentration of particulate material (if known),

   3) dispersing agents and their concentration,

   4) dispersing procedure, including sonication conditions: time, frequency and applied power (if applicable),

   5) viscosity of the suspension,

p) the measurement conditions;

   1) actual particle number concentrations investigated,

   2) temperature of the sample (K) ,

   3) camera type,

   4) camera shutter (ms) ,

   5) camera gain,

   6) frame rate (fps) ,

   7) viscosity (cP) (see 3.15) ,

   8) total frames,

   9) number of processed frames,

   10) number of valid tracks,

   11) calibration (nm/pixel) and its uncertainty,

q) Graphical particle size distribution in either histogram or frequency format. This graph should be suitably weighted and can be subsequently altered by, for example, smoothing or fitting to a model.

r) Measurement uncertainty — this parameter is obtained independently from measuring a reference sample as described in 5.4.

s) All operating details not specified in this document, or regarded as optional, together with details of any incident that can have influenced the result(s).

t) Operators should record all relevant evaluation and measurement parameters. These, however, do not have to be added to the test report.

u) The name(s), function(s) and signature(s) or equivalent identification of person(s) authorizing the test report.

Annex A
(informative)

Theory

This annex gives the derivation of the *n*-dimensional diffusion coefficient that is used[13] to evaluate the size of tracked particles. By definition, a particle flux through point, length or an area per unit time is given by $J(x_n,t)$.

$$J(x_n,t) = c(x_n,t)\upsilon(x_n,t) \tag{A.1}$$

where x_n is the set of coordinates in n dimensions ($n = 2$ for diffusion in the plane of 2D and $n = 3$ for movement in x, y and z directions). Statistical mechanics gives the chemical potential which is related to local probability density (or local concentration) as

$$\mu(x_n,t) = \mu_0 + k_B T \ln\left(\frac{c(x_n,t)}{c_0}\right) \tag{A.2}$$

where $\propto_0$ and c_0 are constants. The variation of chemical potential in space creates a force on the particles given by

$$F = -\nabla\mu(x_n,t) = -\frac{k_B T}{c(x_n,t)}\nabla c(x_n,t) \tag{A.3}$$

Since the velocity of particles $v(x_n,t)$ is proportional to the force applied in the regime of low Reynolds numbers for the liquid, the $v(x_n,t) = \sigma F(x_n,t)$ where constant σ is the mobility of particles.

For spherical particles with diameter d, σ is given by the inverse of the Stokes drag

$$\sigma = \frac{1}{3\pi\eta d} \tag{A.4}$$

According to Fick's law $J(x_n,t) = -D\nabla c(x_n,t)$ where the diffusion constant D is given by

$$D = \frac{k_B T}{3\pi\eta d} \tag{A.5}$$

Assuming that during a normal diffusion process particles cannot be created or destroyed, the flux of particles into a volume shall be equal to that out of the volume. This leads to a continuity formula

$$\frac{\partial}{\partial t}c(x_n,t) + \nabla J(x_n,t) = 0 \tag{A.6}$$

This therefore leads to the following differential formula

$$\frac{\partial}{\partial t}c(x_n,t) = \nabla\left(D\nabla c(x_n,t)\right) \tag{A.7}$$

Assuming that there is no spatial variation of the diffusion constant D, the diffusion formula is recovered

$$\frac{\partial}{\partial t}c(x_n,t) = D\nabla^2 c(x_n,t) \tag{A.8}$$

The above diffusion formula has two main features: it is linear and it is separable. This means that the diffusion processes in three orthogonal directions are independent of each other and the solution to the formula can be written as

$$c(x_n,t) = X(x)\cdot Y(y)\cdot Z(z)\cdot T(t) \tag{A.9}$$

By substituting for $c(x_n,t)$ in the diffusion formula one obtains a separable form for all four dimensions.

$$\frac{\partial}{\partial t}c = D\left(\frac{\partial^2 c}{\partial x^2}+\frac{\partial^2 c}{\partial y^2}+\frac{\partial^2 c}{\partial z^2}\right) \tag{A.10}$$

This leads to the separate form

$$\frac{1}{DT}\frac{\partial T}{\partial t} = \left(\frac{1}{X}\frac{\partial^2 X}{\partial x^2}+\frac{1}{Y}\frac{\partial^2 Y}{\partial y^2}+\frac{1}{Z}\frac{\partial^2 Z}{\partial z^2}\right) \tag{A.11}$$

This form can be further split to demonstrate that the left hand side and the right hand side of this formula can only be equal if they are both equal to the same constant.

$$\frac{1}{DT}\frac{\partial T}{\partial t} = -\left(m_1^2+m_2^2+m_3^2\right) \tag{A.12}$$

This allows the whole Formula (A.11) to be rewritten in a linear operator form

$$\Im = \frac{\partial}{\partial t} - D\nabla^2 \text{ with } \Im\left[c\right] = 0 \tag{A.13}$$

By using the main property of linearity, one can represent the solution to this formula as a superposition of all solutions of the formula. This allows us to use Fourier transform and Green's function methods to show that the diffusion in three dimensions is simply the simultaneous 1-dimensional diffusion process in three orthogonal directions. This statement has the impact on the evaluation of the statistics of particle diffusion and it can be shown that in n dimensions the root-mean-square (rms) motion of the particles can be represented as

$$\overline{(x)} = \sqrt{\left\langle x^{-2}\right\rangle - \left\langle \bar{x}\right\rangle^2} = \sqrt{2nDt} \tag{A.14}$$

This means is that for every unrestricted diffusion dimension there is a $\sqrt{2Dt}$ contribution to the rms value of the particle position. In most PTA measurements the positions of particles in the x and y directions are tracked (in 2D). The rms value is therefore 2-dimensional. Note that the movement of the particles in the sample is 3-dimensional and it is only the projection of that movement that is recorded

during the tracking. Evaluating the rms positions independently in x and y directions allows one to reconstruct the vertical component under the assumption that the diffusion movement and particle statistics is equivalent in all 3 dimensions as it was proven above.

$$d_x = \frac{k_B T}{3\pi\eta D_x} = \frac{2k_B Tt}{3\pi\eta\overline{(x)^2}} \tag{A.15}$$

where d_x is the spherical diameter of the tracked particle due to the x direction of movement. The same result (d_y) should be available from the movement in the y direction.

The assumption of the spherical symmetry of the particle can set a limitation on the applicability of the above derivation to the sizing of particles with aspect ratios different from 1. The statistics of movement of fibres or plates can be different from that of a spherical particle due to the Stokes' drag.

The derivation also assumed that the particles are not created or destroyed during the measurement time, t. This can therefore impose a limitation on the studies of agglomeration, aggregation or dissolution where particles change shape, size or disappear altogether. The key is that the particle state (size or shape) should not change during the observation time, t.

Annex B
(informative)

Apparatus settings and best practice

An interlaboratory comparison was organized to determine the reproducibility of the technique under certain conditions and to develop best practice[11]. The key finding of the interlaboratory study was that by following prescribed protocols the measurement coefficient of variation, repeatability and reproducibility were improved. This was observed even in cases of brief training and short experience history of usage of PTA equipment.

The interlaboratory study can be summarized by the following steps:

Round 1: The 4 monodisperse samples in water without any prescribed protocol

— gold 30 nm

— 100 nm carboxylated polystyrene

— 100 nm aminated polystyrene

— 100 nm silica

Round 2: The second round utilized the same samples as in round 1, but used the protocols described in detail in this document. Three independent video recordings of approximately 60 s were made to enhance the results statistics.

Round 3: The results of the first two rounds were made known to testing laboratories and three new samples were introduced:

— 100 nm polystyrene

— 60 nm gold

— 80 nm gold

The protocols were enhanced by changing the shipping requirements to keep the samples above 4°C, using 0,02 µm filters and HPLC grade water. Six independent video recordings of approximately 60 s were made for each measurement.

Round 4: The samples in this round were a bimodal sample of 80 nm gold with 200 nm polystyrene (of approximately the same number concentration) and a monodisperse sample of 100 nm polystyrene nanospheres suspended in biological media. For sample preparation, the TS user should refer to Reference [11].

The samples were transported with two sensors enabling confirmation that their temperature did not drop below 4°C or rise above 29°C.

The results of the interlaboratory study are summarized in Table B.1 taken from Reference [11].

Table B.1 — Relative standard deviation (RSD) (%) for each sample in the interlaboratory comparison [11]

Interlaboratory comparison round	Round 1 % RSD	Round 2 % RSD	Round 3 % RSD	Round 4 % RSD
30 nm gold	54,8	10,5		
100 nm carboxylate polystyrene	33,3	9,3		
100 nm aminated polystyrene	31,2	15,9		
100 nm silica	34,5	10,0		
100 nm polystyrene			3,5	3,1
60 nm gold			5,1	
80 nm gold			4,1	
100 nm polystyrene and nutrient mix				3,7
100 nm polystyrene and nutrient mix with BSA				4,7
80 nm gold (in bimodal sample)				5,5
200 nm polystyrene (in bimodal sample)				5,2
Average (% RSD)	38,5	11,4	4,2	4,4
NOTE All samples were dispersed in water.				

One of the main conclusions of the interlaboratory study[14] was that by following the best protocol the measurement accuracy is increased significantly as was observed from round 1 to round 4.

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