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Reference methods and materials for counting by instruments using aperture impedance pulse counting (Coulter principle)

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Introduction

Demonstration of valid analytical measurements (VAM) requires reference materials, preferably certified, whose assigned values are traceable to international standards of length or mass.

There are two basic VAM methods. One is to determine a quantity absolutely by means of an accepted procedure in which the errors have been well defined and quantified. In this case the reference materials are those providing traceability of the procedural components, eg. certified balance weights.

The other is to run a simultaneous comparison of the unknown sample with a calibrator. Few devices other than eg. a chemical balance, truly compare two things simultaneously, but for the majority of stable analytical techniques the meaning has been stretched to mean "close together in time". Most haematological instruments are comparitors falling into this category. Although there are now established reference materials and techniques for particle and cell sizing, there are as yet none, other than counting by eye, by which values of number can be certified.

One reference material exists, NIST SRM 1003 glass ballotin [4], with number versus size distribution certified by counting and sizing under the microscope by an internationally accepted body. Only the relative number distribution is given however, the number of beads per gramme of SRM must be computed from the (uncertified) relative density for the glass ballotini. It is also well known that such counts, being very low, have intrinsically poor precision, making them unsuited for the number calibration of count comparison instruments whose results may be several orders of magnitude higher.

Because of this lack of primary references, manufacturers of comparitor instruments usually provide alternative blood based reference materials commercially. Even here, however, apparently only two haematology products claim to be calibrators, ie. to be assayed by traceable techniques, Coulter Scal and Streck Corporation's Cal-Chex.

Latex suspensions are obtainable commercially, with extremely narrow particle size distributions either as single populations (Fig. 1) or mixtures of these, eg. Accubeads from the Fastecs division of Kodak. Mixtures of different bead sizes may cover a very wide overall size range (Fig. 2). Normally, only approxi-

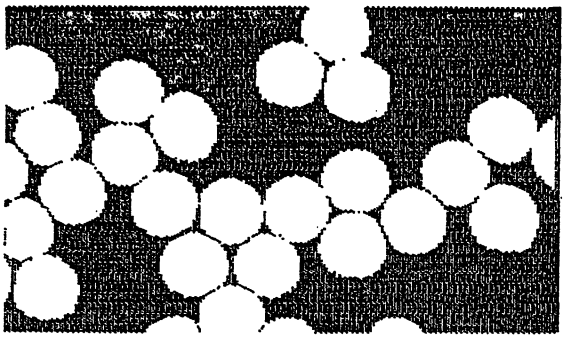

Fig. 1. Micrograph of a single population of latex beads with an extremely small c.v. (Fastec 2.5 µm Accubeads at 2500 x)

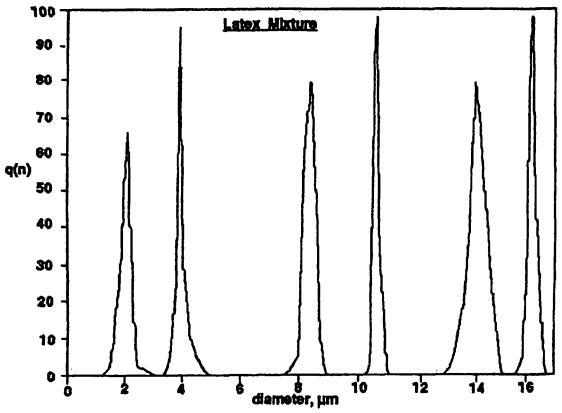

Fig. 2. A mixture of six latex bead populations each with a small c.v. The relative proportions of each component may be given, but the absolute concentration is unknown

mate concentrations of particles are quoted for these. Like the SRM 1003 assays, relative proportions are given rather than absolute counts.

Coulter SpheriCal and Becton Dickinson Corporation's FacsCount both have accurate particle concentration values assigned for use as cytofuorometer calibrators.

Definitions: The terms accuracy, uncertainty, etc.., used here are based on the definitions given by British Standards [1] and ISO [2, 3].

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Latex suspensions are notoriously difficult to keep stable. Aggregation can cause irreversible loss of counts with time and, unlike blood samples with an extensive background of investigation into optimum storage conditions, little is known about how to stabilise latexes as concentration calibrators. A BCR programme to produce count standards proposes dispersion of „monosized" latexes in a protein matrix followed by freeze drying to achieve long term stability.

Two other factors limit the use of non-blood based count calibrators to essentially non-haematological instruments such as cytofluorometers, one being the importance of matrix effects, the other the relative cost. Most automated haematological instruments have sampling systems that are specifically designed to aspirate and dilute blood specimens. Presented with a non-blood based product, they may well make final analytical suspensions that are incorrect.

These effects are so important that they cannot be ignored even for blood based calibrators. The procedure shown schematically in Figure 3 illustrates how the problem can be avoided by performing whole blood calibrations on a non-comparitor instrument, using these to calibrate a comparitor instrument, then assigning values to reference materials on the comparitor. For Coulter Scal, in fact, assays are also carried out on both types of instrument; they are not expected to differ by more than the allowed tolerance for the assay value.

One potentially attractive alternative technique, not yet fully investigated experimentally, is the validation

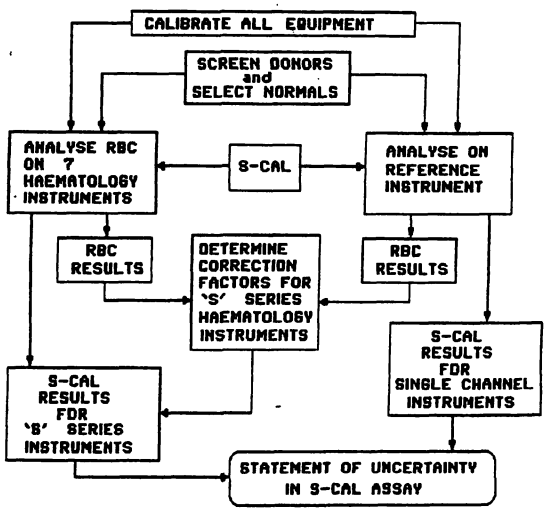

Fig. 3. Schematic representation of whole blood calibration for assigning reference values to SCAL blood based calibrator. This scheme takes into account any matrix effects and conforms to ICSH calibration protocol

of counts of a suspension of ultra-narrow (coefficient of variation < 1 %) latex beads with known mass concentration. The theoretical mass of a single bead is determined by calculation from its independently certified diameter and the precisely known material relative density. The accuracy with which a number can be assigned this way is always limited to approximately three times that of the diameter, eg. if a bead diameter is only known to 1 %, its mass cannot be assigned to better than = 3 %.

There have been several proposals [5–7] for directly assigning instrument assays to cell based calibrators. The proposed BCR count standards, mentioned above, also involved assays by particle counter, but the tolerances quoted for the certified values were more than wide enough to enable non-comparitor instruments to be regarded as accurate devices in their own right.

Evaluation of uncertainty

An electronic counter that is not a comparitor can be used as a count assaying device by determining its error budget and qualifying the assigned value by the total uncertainty associated with the measurement [3, 8].

Ideally a reference material to be assayed from first principles should be a pure particle suspension. Real samples can then be "spiked" with an accurately known amount of the reference for use in comparitor instruments. This technique is widely used in other disciplines such as chromatography to provide internal standardisation and is the method of use for Coulter SpheriCal.

Directly assigned counts: Erythrocytes, leucocytes and latexes

Erythrocyte and leucocyte whole blood reference assays can usually be achieved directly from first principles because the high relative concentration of the former minimises the interference from other cell populations, and the latter are well differentiated by being nucleated. Strictly speaking, reference values for erythrocytes would be more accurately measured by removing all interfering cells, eg. by passing the source sample through leucocyte and platelet removing filters, provided this did not cause other problems. In practice, pre-analysis of all source material is essential to ensure suitability for both blood based and latex calibrators.

Indirectly assigned counts: "Spiking"

Directly counted assays of overlapping cell populations are harder to obtain than single particle populations. One deconvolution technique obtains counts not by discrimination between size limits, but by curve fitting to partial size distribution data, then calculating the area under the entire fitted curve. This technique is used in Coulter automated haematology instruments for platelet counting because it caters for wide thrombocyte size distributions and ensures close agreement between the current reference method (phase contrast microscopy) and instrument results.

Once a suspension of particles has been assayed, it can be added in accurately known amounts to analytical samples so as to provide an internal concentration reference for each one. For example, an ICSH proposed instrument based reference technique determines platelet counts by the ratio of their counts between specified size limits to the RBC values in the same samples using a comparitor instrument. The erythrocyte population in this case represents the internal quantity marker. Platelets outside the limits are excluded. The RBC count is determined elsewhere.

An alternative, potentially more accurate, calibration technique after Anderson [9], uses platelets separated from whole blood as the "spike". Concentration is determined as a solitary population by direct counting in a non-comparitor instrument between reference sizes. This count can, if necessary, be linked to be fitted curve method by comparing it with the area under the curve bounded by the same size limits (Fig. 4). Whole blood is counted on the instrument to be calibrated, firstly before the platelet concentrate is added quantitatively, then after.

The platelet calibration factor, f, for a comparator instrument (CI) is given by

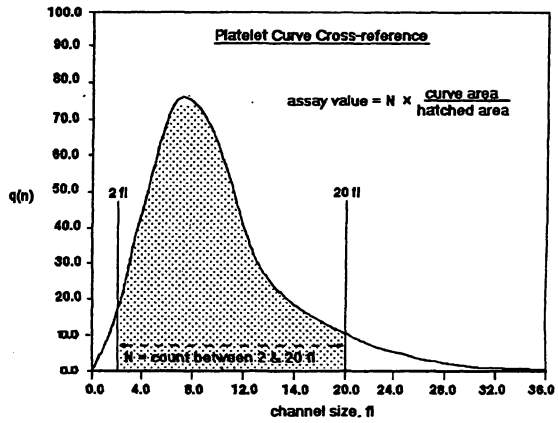

Fig. 4. Method for cross referencing platelet counts between size limits to fitted curve results. Once platelet-rich plasma has been counted, it can be used to "spike" (add volumetrically to) samples as an internal count standard for analysis in comparitor instruments that integrate platelet curves to arrive at platelet concentration. Once calibrated, such instruments can then assign values to calibrators

$$f_t = \frac{P_1(v/(v+V))}{P_3 - (P_2(v/(v+V))}$$

Where
P_1 = Platelet rich plasma (PRP) reference count
P_2 = Whole blood platelet count from CI
P_3 = Spiked sample platelet count from CI
V = Volume of PRP used
v = Volume of blood used

Spiking also allows quantitation of specific types of cells (eg. CD4 + lymphocytes) in flow cytometry. Coulter SpheriCal latex particles with known concentration are added quantitatively to the sample before aspiration into the flow cytometer. Because cytometers usually use more than one parameter to distinguish between cell types, complete separation of cell populations from latex does not present any undue difficulty.

Counts of cells are then computed in the same way as platelets, above, but substituting latex concentrations for P^1, P^2, P^3. The uncertainty in this kind of enumeration has not yet been evaluated. One potential source of error that must be guarded against is that the reference count may be of all particles of a certain sized population, but a flow cytometer may be set to respond to some other characteristic of that same population and give a different count.

For example, in a population of fluorescent monosized latex particles, there may be a distribution of fluorescent moiety within that population or a portion of the beads may not fluorescense at all. Counting all particles in the sample may give a misleadingly high value for the expected response in the cytofluorometer. It is essential that, just as with pure count references, the source material be pre-analysed both as a pure population, and added to a typical sample matrix, to determine the degress of agreement between parameter counts.

Breaking down an analysis, shown schematically in Figure 5, into components and assessing these for sources of error, we can try to compensate for systematic errors and to minimise random error at each stage. This keeps the total uncertainty as small as possible.

Many of the requirements given here are also factors that should be considered when using count control material particularly the effect of matrix effects on sampling, and losses due to sample vial wall adhesion and settling.

My proposals here deviate in some respects from the current ICSH draft reference (Draft 4, Jan. 1993).

Source material

We select only whole blood samples having no abnormal properties that will interfere with accurate counting, and latex beads that have a single, monodisperse, population.

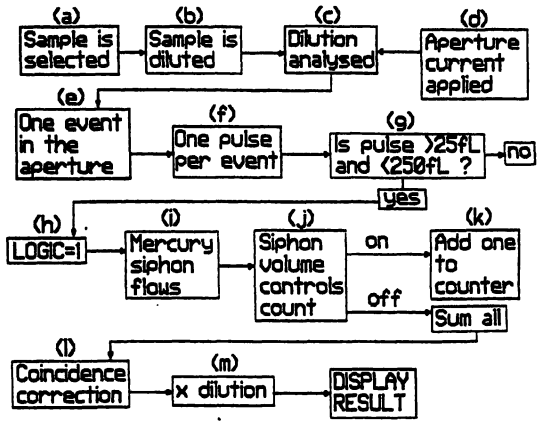

Fig. 5. Schematic representation of count analysis. Treating analysis as a sequence of modules allows close inspection and auditing of sources of uncertainty. Only systematic error of known dimension can be compensated for

Dilution

We use bulk dilutions (eg. for RBC, 1 ml in 500 ml, then 1 ml in 100 ml to achieve a required 1:50,000 final dilution). Pipettes, preferably Kirk pattern, modified to be usable with a pipette filler, are calibrated by analytical balance to contain. Flasks are certified. Pipettes are always used by washing their contents out. With Kirk pipettes it is possible to achieve an accuracy compared to gravimetry of ± 0.4 %. A very careful technique is needed to ensure that no undue stress is applied to erythrocytes during aspiration and dilution.

Beakers

We have found that analysis of cell and particle suspensions is best made in acid washed glass beakers with gentle agitation. Lack of stirring appears to be the major cause of decrease in latex and cell counts over time, Figures 6a and 6b, though the presence of certain plastics seems to contribute to count loss.

Beakers should be rinsed with a small amount of sample suspension immediately prior to analysis of the sample.

Aperture Current

Excessive current can damage red cells during transit through the aperture, caused when the voltage stress across the cell exceeds the reversible potential of the

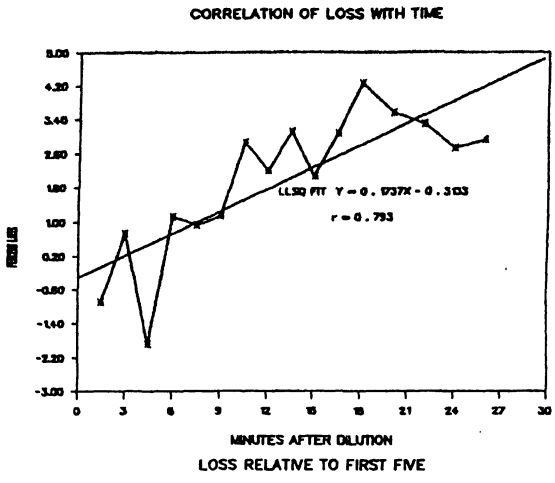

Fig. 6a. Loss of count increases with time in an unstirred beaker.

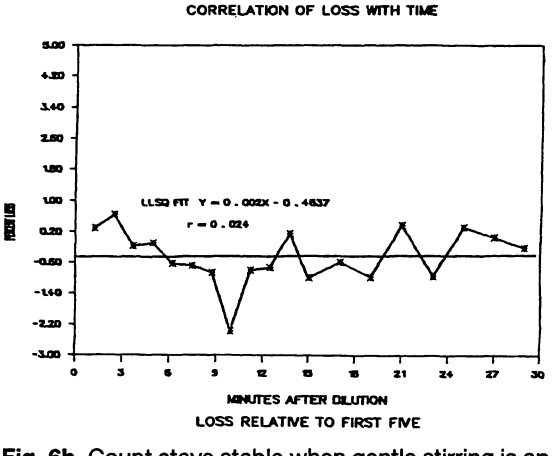

Fig. 6b. Count stays stable when gentle stirring is applied.

membrane [10], approximately 1V. W can calculate the limiting current for an aperture given its dimensions and the size of the cell. Alternatively, it can be determined empirically by plotting apparent mean cell volume (MCV) against applied current, Figure 7. A working current should be less than the drop off point. I use an upper limit of 700 µA for a 100 µm diameter aperture and 300 µA for a 50 µm aperture.

Damage is also thought to occur when red cells are exposed to the products of electrolysis for a period of time, though my own measurements in stirred glass beakers show that this is not completely true; RBC suspensions are sufficiently stable to allow in excess

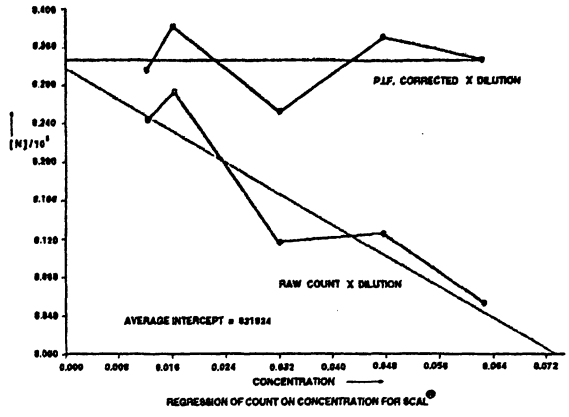

Fig. 7. I) Graphical method for correcting for coincidence (after Thom). Counts corrected for dilution are plotted against concentration and extrapolated back to zero concentration. The intercept (a) on the ordinate represents the true count. Factor P for use in Princen and Kwolek's quadratic coincidence correction equation is given by $P = \tan 0 / a^2$
II) Plot of time-of-flight corrected counts treated in the same way. The results are uncorrelated with concentration (tan O = 0)

of ten counts to be made over a period for several minutes without visible change, providing that the beaker contains sufficient diluent volume, eg. in excess of 50 ml. Techniques exist to flow suspension to the aperture in such a way that the bulk of the stirred sample is not exposed to electric current.

Losses

Dilutions and aperture sizes should be chosen to try to ensure that multiple particle passage is minimised. The usual haematological dilution of 1:50,000 on a 100 µm aperture tube gives rise to over 10 % loss of RBC count. More accurate results can be achieved by using higher dilutions and counting more suspension and/or using a smaller aperture.

Equivalence of pulses to particles

One of the reasons why instrumental counting is not obviously a substitute for counting by eye is the difficulty in proving equivalence between one event in the aperture and one pulse in the counting circuit. Although electronic counters can in theory be validated with traceable pulse generators, these do not reproduce the random nature of the aperture pulses.

Because apertures must obey both Ohm's and Kirkchoff's laws, there is no possibility, other than a

malfunction, of particle or multiple particle passage not generating a pulse. Noise, interference and recirculation of particles can all cause spurious counts. These must be absent or insignificant during analysis. The height of recirculation pulses is usually limited to 4 % of that of pulses from aperture transit. The design of automated haematology instruments and cytofluorometers usually precludes particle recirculation.

Count settings

Counts of monodisperse latex materials are normally made at the pulse half-height, ie. at essentially three-quarters of the latex particle diameter. Counts of 10 µm latex would therefore be made at 7.5 µm and so forth. This count will be relatively constant over a range of size, but losses due to coincidence are dependent on the pulse width at the counting threshold [11]. Counts taken of latex particles in a cytofluorometer for example with the gate set down near the base of the pulses may well be much lower than the assay value.

Routine counts of RBC are usually made at a lower relative setting than half height, above 25 fl, so that microcytic RBCs are not missed. For reference work this is not relevant since pre-analysis should exclude bloods containing these from being used as calibrator source material. Reference RBC counts on instruments not specifically designed to exclude recirculation are best taken above 35 fl.

Table 1. Results from two sets of analyses 25 fl with and without aperture rear sweep flow to remove recirculation

	with	without	% difference
Mean A	28151.4	28351.0	-0.7
Mean B	28356.3	28465.5	-0.4

Poisson expected c.v. = 0.6 %.

Gating the pulse train

While pulses are being counted a precise volume of sample must be metered through the aperture. There are several considerations here.

We can trace the volume of our mercury siphon to the chemical balance. The siphon volume can be traced by means of an oil immersed piston mounted on a micrometer via a hydraulically rigid system to displace mercury weighed on an analytical balance. The displacing device piston diameter has also been gauged with an National Physical Laboratory (NPL) traceable micrometer. The balance is itself checked and calibrated with weights traceable to NPL of 100, 50, 10, 1, 0.1 and 0.01 g. All mercury displacements, typically 6g Hg, are weighed to better than 0.1 mg, ie. to better than 0.002 %.

Table 2. Certification of the volume of two mercury siphons by means of a traceable device

Measurement	Siphon Serial No. 54440	Siphon Serial No. 58980
1.(Unwashed) Static method	100.48 µL ± 0.43 503.64 µL ± 0.48	101.19 µL ± 0.43 504.22 µL ± 0.43
2.(Unwashed) "on the fly" method	100.13 µL ± 0.07 503.48 µL ± 0.07	100.94 µL ± 0.08 503.81 µL ± 0.11
3.(After acid washing) Static method	100.76 µL ± 0.59 503.24 µL ± 0.50	101.16 µL ± 0.56 504.22 µl ± 0.45
% differences between 1 and 2	0.350 % 0.032 %	0.248 % 0.081 %

The calibrations of the displacement device by length (gauge) and balance (Hg displacement) agree to within 0.016 %. An angular encoder attached to the (motorised) piston allows the calibration to be performed with flowing mercury (on the fly) with a sensitivity better than 0.131 µl (0.026 % of 500 µl). The meniscus can also be adjusted by hand (static method). Both methods agree to within 0.2 % on a conventional siphon and can be less than 0.03 % on a specially constructed one. Most of the 0.2 % difference appears to be in shaping the meniscus as the mercury flows. The device's measured imprecision on the fly is typically less than 0.015 %.

Both precision and accuracy can be improved by arranging for all electrical siphon contacts to lie in the vertical plane so that the mercury meniscus is horizontal. Accuracy can als be improved by utilising a longer, narrower, capillary than normal but this limits the number of volumes to two. The overall uncertainty in a calibrated siphon can be assessed as 0.2 %.

Constructing the entire aperture-siphon system out of rigid glass without flexible plastic tubing increases hydraulic rigidity. Use of 15 mmHg vacuum causes outgassing of diluent. Water generally dissolves about 3 % air at room temperature and some 30 ml of diluent are normally needed to prime a counter. By experiment, about 20 µl of air are outgassed during the first three minutes at a siphon head of –150 mmHg. Equilibrating diluent in a vacuum chamber before filling avoids this.

Curve fitting the data of Table 3 to an exponential ($r = 0.997$) gives a value of about 3 µl of air outgassed in 50 s (for the 50 x 60 aperture measurements) and about 1.8 µl in 12.5 s (for the 100 µm aperture). These correspond to systematic errors of 0.6 % and 0.36 % respectively.

Table 3. Times for cumulative gas evolution from Isoton II at 23°C and -150 mmHg

Volume, µl	time taken, seconds
20	169
40	215
80	265
120	281

If siphon flow times are known for outgassed diluent, the values for ordinary diluent can be used to correct the volumes metered for their systematic bias. The need to use outgassed diluent for counting can then be avoided.

Gas is also electrolytically released at the inner electrode. The volume rate can be calculated using the gas laws from the aperture current and the faraday. The rate can also be measured experimentally by constructing a special „no aperture" aperture tube in which the electrodes are both on the inside. The proportions of gas are, of course, always 2:1 by volume.

Calculation shows that a change of 1° C leads to a volume change of about 6 µl for the 30 ml filling the interior of an aperture/siphon = 1.2 % volume error in a 500 µl siphon volume. The thermal inertia of the glass and liquid, however, should ensure that even in the most wildly variable conditions, fluctuations only occur on a long time scale. Analytical samples and internal instrument diluent ought to be closely similar in temperature.

Coincidence correction

Most corrections start from Poisson assumptions and conclude with formulae that are applicable only to the dilute state eg. less than 5 % loss. Naturally, it is the higher concentrations that have the greatest need of accurate correction and for which the Poisson assumptions are least likely to be valid.

Reference 6 proposes a graphical method for correcting counts without presupposing any particular theory. The instrument count corrected for dilution is plotted against volumetric concentration, a straight line fitted, and extrapolated back to zero concentration to give the true value, Figure 8. This is a far more time consuming operation than solving equations and requires a number of dilutions of equal accuracy to be made. A more serious objection is that a separate graph needs to be constructed for each combination of circumstances.

It is not difficult to show, however, that this method is actually identical to the solution of Princen and Kwolek [12] based on the binomial expansion. In their equation $No = N - pN^2$ where No is the observed or instrument count of particles, N the true count, and p a factor relating to the dimension(s) of the aperture,

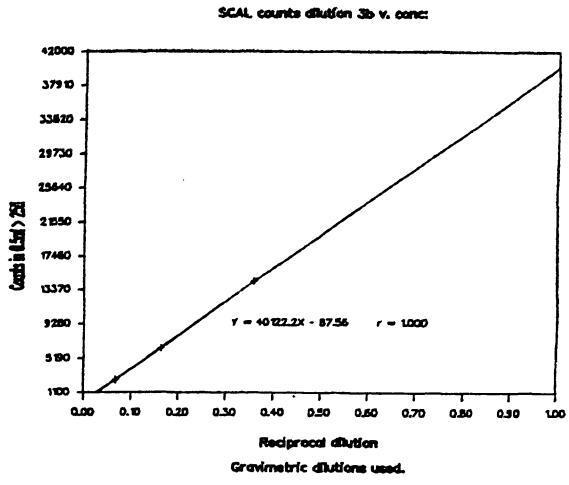

Fig. 8. Plot of time-of-flight corrected counts for dilutions covering a 20:1 range of concentration, showing perfect correlation between the results and the concentration. The intercept is in excellent agreement with the average blank count obtained immediately prior to analysis (45 particles per ml)

p is given by the ratio b/a^2 where b is the slope of the graphical method, and a its intercept in the Y axis. Both have the additional difficulty that linear least squares fitting emphasises higher ordinate values. These are the products of lower counts and higher dilutions, both of which carry the highest uncertainty. As a consequence, considerable care must be exercised in carrying out the extrapolation.

Harfield, Lloyd and Cowan have shown [11] that there is a more fundamental method of determining the loss of cells, free from prior knowledge of aperture dimension, analytical volume, dilution, size setting or sample, and which, unlike the above methods, is monotonic. The proportion of time occupied by pulses in the counting circuit, whether from single or multiple events, can be shown to a function of the true particle concentration. This method can be referred to as „time of flight correction", TOFC.

TOFC requires its own dedicated circuitry and is therefore only to be found in the newest models of particle counter such as the Coulter Z1. It can be used directly to correct instrument counts or indirectly to derive the p value for the Princen and Kwolek equation. This latter method is currently implemented in the latest version of Coulter model Zm internal software.

TOFC is ideally suited to correcting counts of small particles in the presence of large, such as occur in „spiked" assays.

Table 4 shows a comparison between ZM results corrected by TOFC using a 100 µm aperture, and results from a special 50 × 60 µm reference system in which uncertainties have been minimised or quantified as above. Differences in mean counts are closely similar

Table 4. Comparison of TOFC corrected counts (1:50,000 dilutions) on a 50 x 60 µm aperture "reference" system with those from a conventional Sampling Stand fitted with a 100 µm aperture

Sample #	50 x 60 µm Mean TOFC	50 x 60 µm Mean Raw	100 µm Mean TOFC	100 µm Mean
Count data				
1	47568.4	48313.2	43947.5	48081.2
2	47278.0	48250.7	44099.0	48359.0
3	47842.0	48848.0	44269.5	48705.3
4	48486.8	49104.5	4465.3	49141.4

Coefficients of variation of the raw counts and relative differences of the means.

	C.V.%	% Differences 50 x 60-100	C.V.%
1	0.331	0.480	0.501
2	0.993	-0.224	0.571
3	0.430	0.292	0.197
4	0.068	0.759	0.417
Averages	0.456	0.327	0.422

to raw count imprecision. Figure 8 shows TOFC results for SCAL material plotted for comparison with the „graphical" method. The high degree of confidence available from TOFC results is shown in figure 10 where corrected SCAL counts are linearly regressed with r = 1.000 against gravimetric dilutions in a concentration range ov 20:1. The intercept of 87.56 particles at zero concentration is not significantly different from the blank count of 45 particles (the latter was not included as a regression point).

Random Errors in Counting

Random count scatter is usually described by Poisson statistics which are really only applicable to very low event probabilities. Where counts of 80,000 ml^{-1} are being obtained with a 100 µm aperture, the probability of an event is about 0.1. Space does not permit justification at length but I propose the following formula for the expected precision of counts.

$$c.v. = \frac{100}{\sqrt{[1+1/j)/n]}}$$

where n is the mean observed count and j is the number of contributing counts to n.

Values from this equation can be used in single sided variance ratio tests with j-1,j-1 degrees of freedom.

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Laryngeal Diphtheria due to toxigenic Corynebacterium ulcerans

In march 1993 we diagnosed laryngeal diphtheria [1, 2, 3] due to toxigenic C. ulcerans in a 42 year old jobless woman (81 kg, 156 cm) with a diphtheria antitoxin concentration in her serum of 0,01 IU/ml by PHA[1]. As a child she had been vaccinated against diphtheria toxin.

After a long history of bronchial asthma she was admitted to our hospital because of deteriorating dyspnea after two weeks of coughing and expectoration of whitish material and an asthma attack the day before admission. The only medication she took was salbutamol spray. On admission body temperature was 36,8 °C, blood pressure 90/60 mmHg.

Clinical chemistry data:

Leukocyte count	35 000/µl
MCV	87,9 fl
Gamma-GT 100 U/l	(18 U/l)
CK-NAC 150 U/l	(10–70 U/l)
CK-MB 13 U/l	(<10 U/l)

One day after admission dyspnea deteriorated rapidly, so she was intubated. The tonsils and the swollen larynx were covered with a robust yellow-whitish pseudomembrane extendeding well into the upper trachea.

After intubation through the reduced lumen of the larynx a pseudomembrane 2 × 3 × 0,15 cm in size was expectorated.

The next two days an iv dose of 40 000 IU and in dose of 60 000 IU of diphtheria antitoxin were given together with four mega penicillin daily for ten days.

The presumptive microbiological diagnosis of diphtheria was reported to the hospital 30 minutes after the arrival of the pseudomembrane in the laboratory, the positive Elek test 48 hours later.

Neisser's stain of the pseudomembrane's smear was packed with typically coulored rods.

Gram stain of the pseudomembrane smear showed gram-positive and gram-labile "diphtheroids" and neutrophil granulozytes.

C. ulcerans was easily grown from the membrane but nasal and pharyngeal smears taken at the same time and transported less than 30 minutes in Portagerm transport medium (bioMerieux) were negative. API Coryne (bioMerieux) was used for biochemical identification.

MIC's (mg/l) determined by a commercial microdilution technique[2] were as follows:

Penicillin G:	< 0,25
Amoxicillin:	< 2
Azlocillin:	<16
Erythromycin:	< 1
Cephalexin:	< 4
Cefuroxim-Axetil:	< 2
Cefixim:	1
Gentamicin:	< 1
Cotrimoxazol:	<16
Tetracyclin:	< 1
Ofloxacin:	< 1
Chloramphenicol:	< 8
Clindamycin:	< 1

The toxin assay in the guinea pig was also positive[3]. On her third day in hospital laryngoscopy still showed massive laryngeal swelling. Pseudomembranes extended down the trachea and well into the central bronchial tree. Cervical soft tissue and lymphnode swelling developed gradually.

On day 12 the circumference of the patients neck which normally was 38 cm reached a maximum of 50 cm. On day 15 she was extubated but had to be tracheotomized due to severe stridor.

After 41 days including 25 days of intensive care she was decanulized. One week later she was deferred presenting with no residual symptoms apart from her tracheostoma. The electrocardiogram was normal.

She had drunk raw milk several times 6 to 9 months before she became ill [4]. The farmer from whom she had bought the milk treats his animals without the help of a veterinarian. She had a dog, a hamster, a guinea pig and a rabbit in her house. Pharyngeal smears of contact persons including personnel of our intensive care unit did not show C. ulcerans. Contact persons without immunity against

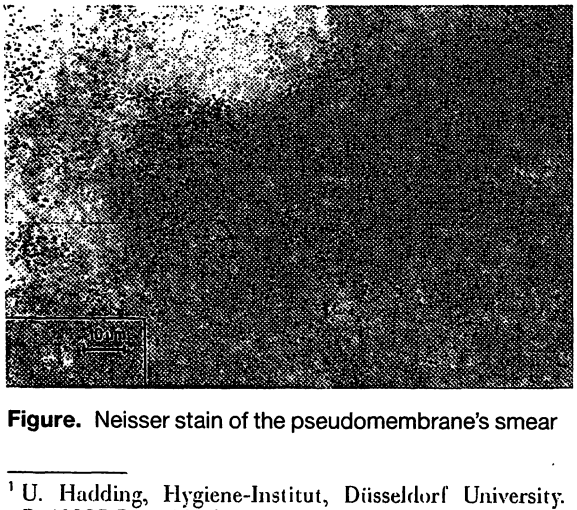

Figure. Neisser stain of the pseudomembrane's smear

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