A TWELVE-CHANNEL AUTOMATIC DEVICE FOR CONTINUOUS RECORDING OF CELL AGGREGATION BY MEASUREMENT OF SMALL-ANGLE LIGHT-SCATTERING

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SUMMARY

We describe here a 12-channel aggregometer, evolved from the instrument described by Beug & Gerisch in 1972, which records the course of aggregation or agglutination of cells or other particles by following the changes in light-scattering of the aggregating suspension. The instrument incorporates a simple memory system for condensing the data, introduces an improved cuvette design, and can be adjusted in its responsiveness to light-scattering by particles of different sizes.

In our aggregometer, a vertical wheel in an opaque thermostatted chamber accommodates up to 12 cuvettes each containing a cell suspension and an air bubble. The constant rising of the bubble as the wheel rotates causes the stirring action which promotes aggregation. At a certain point in its rotational path, each cuvette is penetrated by a beam of light focused upon an absorbing beam stop centred in front of a photomultiplier tube. Particles suspended in the cuvette scatter a portion of the light beyond the beam stop into the photomultiplier tube to produce an electrical pulse proportional to the photon input. Collection of these pulses with the wheel in continuous motion avoids any disturbance of the course of aggregation. The pulses are routed to memory circuits for summation and eventual recording by a 12-channel printing potentiometer which automatically colour-codes and numbers each curve.

The cuvettes consist of 2 glass microscope coverslips affixed with silicone grease over a hole in a thin, stainless steel blank. They are very durable and are easily dismantled and reassembled for cleaning. The coverslips are replaced after each use. The discoid chamber of our cuvette permits uniform circulation of the bubble, which in turn causes gentle stirring of the aggregating suspension at a rate that is a direct and continuous function of rev/min.

Measurement of light-scattering at small angles (3.5°) provides great sensitivity to the disappearance of single cells and progressively less sensitivity to the coalescence of aggregates of progressively larger size. The signal generated by the photomultiplier tube decreases as aggregation proceeds.

At 32 rev/min, one data point per channel, representing the sum (average) of 24 successive measurements, is printed every 45 s. In the course of a 60-min assay under standard conditions, up to 23040 individual readings can be automatically taken, summed and plotted. In a comparison of 8 identical samples run simultaneously, the printed values after 60 min agreed with a standard deviation of ±2%.

INTRODUCTION

Investigations of the adhesive and other surface properties of cells often require measurements of spontaneous cell aggregation or of cell agglutination by antibodies, lectins or other surface-acting factors. Various parameters have been assayed for this purpose.
The extent of aggregation in stirred suspensions of dissociated cells has been determined by measuring the final sizes of aggregates (Moscona, 1961; Humphreys, 1963; Lilien, 1968; Gershman, 1970). The rate of aggregation in such suspensions has been measured as a function of either the total number of particles (by direct counting: Curtis, 1969; Edwards & Campbell, 1971; by Coulter-counter determination: Edwards & Campbell, 1971; Takeichi, 1977) or the disappearance of single cells (by direct counting: Curtis & Greaves, 1965; by Coulter-counter determination: Ball, 1966; Orr & Roseman, 1969).

Attempts have been made to minimize various disadvantages of these methods by separating aggregates from unaggregated cells before sampling (Deman & Bruyneel, 1973), by combining Coulter-counter results with particle-size analysis (Whur, Koppel, Urquhart & Williams, 1977; Perry & Jones, 1978), and by counting the number of cells in each particle in samples taken from suspensions of aggregating cells (Armstrong, 1966; Steinberg & Granger, 1966; Steinberg, Armstrong & Granger, 1973). These modifications permit more sensitive, detailed analysis of aggregation, but they are slower and may be impractical when many samples must be assayed.

Rates of adhesion have also been determined by measuring the number of single cells collected from labelled cell suspensions during fixed time periods by cell aggregates (Roth & Weston, 1967; Roth, 1968; Cassiman & Bernfield, 1976; McGuire & Burdick, 1976; McClay, Gooding & Fransen, 1977), tissue fragments (Barbera, Marchase & Roth, 1973; Gottlieb & Glaser, 1975), fibre-bound cells (Edelman & Rutishauser, 1974) and cell-coated beads (Vosbeck & Roth, 1976). These approaches permit measurement of adhesion between cells from different tissues. However, they too are time-consuming, produce few data points and may leave only cell surfaces of a selected kind available for adhesive interactions. Beug & Gerisch (1972) introduced an aggregometer which monitors the change in optical density with aggregation simultaneously in each of 20 samples. Their machine has advantages over earlier designs (Born, 1962a, 1970; Cuthbertson & Mills, 1963a, b; Cunningham & Hirst, 1967; Mills & Roberts, 1967a, b; Michal & Born, 1971) which could handle only a single sample of greater volume. We describe here a 12-channel aggregometer which, while similar in general plan to that of Beug & Gerisch, differs in the following respects. (1) The cuvettes are better suited for assaying avian and mammalian cell aggregation and are readily assembled and disassembled for cleaning. (2) Sensitivity to the appearance and disappearance of particles in different size ranges can be adjusted. (3) Readings are made and automatically recorded essentially continuously without disturbing the stirring of the aggregating suspensions.

**Description**

**General**

A vertical wheel in an opaque, thermostatted chamber accommodates up to 12 cuvettes, each of which, when loaded through special ports, contains a cell suspension and an air bubble. As the wheel rotates, driven by a constant speed motor, the bubble in each cuvette moves constantly toward the top of the chamber, stirring the cell
A 12-channel recording cell aggregometer

suspension and promoting cell aggregation. At a certain point in the wheel’s rotational path, each cuvette is traversed by a beam of light focused upon an absorbing beam-stop centred in front of a photomultiplier tube. In the absence of cells or other particles, essentially all the light passing through the cuvette is absorbed, so that none enters the photomultiplier tube. However, cells or other particles suspended in the cuvette scatter a portion of the light, which spreads beyond the beam-stop into the photomultiplier tube to produce an amplified electrical output proportional to the photon input. As aggregation proceeds, the intensity of this signal diminishes. By means of switching and storage devices, the pulses from each cuvette are sorted out, accumulated, and then recorded on a moving chart by a 12 channel printing potentiometer (Fig. 1).

Fig. 1. Aggregometer: schematic diagram.

Optical system

The light source is a 6-V d.c. lamp powered by a constant output transformer with variable controls. It is used at a fixed voltage to maintain constant colour temperature, and the beam intensity is regulated with neutral density filters. Wavelength is selected using colour filters. In order to minimize background scattering, the beam is tightly collimated before being focused through the plane of the cuvette onto the beam-stop. It passes through the sample with a cross-sectional area approximately 25% that of the cuvette, ensuring that a relatively large fraction of the suspension is ‘seen’ during each pass. The scattered light is measured by an RCA X12-123 photomultiplier tube powered by a constant high-voltage source with variable sensitivity controls. Concentric annular masks and beam stops are used to limit respectively the outside and
inside diameters of the exposed section of the photomultiplier tube face. The measuring angle $\theta$ is determined by the combination of beam stop and annular mask selected (Fig. 2).

**Fig. 2.** Measurement of light scattered through angle $\theta$ (see Fig. 1).

**Mechanical system**

Motor speed is regulated through a control unit equipped with a soft start and stop feature to avoid high transitory shears and a tach-generator feedback circuit to ensure constant speed. A series of switches can stop the cuvette wheel in any of 12 positions for immediate access to any cuvette.

The cuvette wheel accepts 12 interchangeable cuvette holders which are positioned with set screws and are designed for easy insertion and removal of cuvettes. Each cuvette holder has a replaceable aperture which sets the diameter of the beam before
A 12-channel recording cell aggregometer

it enters the sample. Holders accommodate either a single cuvette or 2 cuvettes in tandem (Fig. 3).

The cuvette wheel rotates to bring each cuvette into the light path once per revolution. The beam aspect on each moving aperture waxes and wanes during each transit like the moon's profile eclipsing the sun, producing a sharp, symmetrical pulse as seen on an oscilloscope. The wheel and photomultiplier tube face are enclosed in a light-tight box, the temperature within which is thermostatically regulated by means of a forced-air heating and cooling system.

On the shaft of the cuvette wheel are mounted 12 adjustable cam switches (1 per channel) that route each photomultiplier tube pulse to the appropriate memory circuit. A thirteenth switch advances the revolution counter, which in turn governs

Fig. 3. Cuvette (a), holder and wheel assembly (b) showing interchangeable single (c) and double (d) cuvette holders.
the 12-channel printer's print-command switch. After each point is recorded, the printer automatically advances to the next channel.

**Cuvettes**

Cuvettes are assembled using stainless steel blanks (30 mm x 30 mm x 2 mm with a 20-mm hole in the centre), square no. 1 microscope slide coverslips (25 mm x 25 mm), and silicone stopcock grease (Fig. 3). Each blank has 4 ridges on each face which together form a recess to accommodate a coverslip. When assembled, each cuvette has a discoid interior chamber approximately 0.63 ml in volume, which is accessible through 2 small ports bored in one edge of the blank. These ports are fitted with Teflon plugs with tips milled to match the contour of the cuvette interior. An air bubble of approximately 0.08 ml stirs the cell suspension in each cuvette as the wheel rotates. Without such a bubble no stirring, and hence no aggregation, occurs.

**Memory circuits and printing potentiometer**

The memory circuit is a resistor-capacitor system that receives 1 pulse each time the corresponding cuvette intercepts the light beam. The capacitor sums these pulses until the preset number have accumulated, when it is read and then discharged by the printing potentiometer. There are 12 memory circuits (1 per channel), each of which has a zero-adjusting variable resistor for equalizing the circuits in the absence of an input signal ('dark current'), an equalizing variable (time-constant) resistor for standardizing the channels in the presence of a constant input signal, and a zero-offset variable resistor for adjusting the channel's zero position on the strip chart. The time-constant resistor varies the percentage of each pulse reaching the capacitor, thereby altering the slope of the charging function and of the aggregation curves for that channel. The zero-offset changes the y-intercept of a curve by adding a selected voltage to each pulse from that channel. It does not alter the slope of either the charging function or the aggregation curves plotted from that channel.

An adjustable, automatically resetting revolution counter generates a pulse after a pre-set number of revolutions (usually 2) of the cuvette wheel. This pulse causes the printing potentiometer to read and then print the voltage accumulated in a given capacitor, discharge the capacitor, and finally move to the next channel, where the cycle is repeated at the next pulse.

The printing potentiometer (Philips PM8235) is a 12-channel strip chart recorder which automatically colour-codes and numbers the curves from each channel for easy identification. Full scale can be varied from 0.01 to 5.0 V, and other controls regulate chart speed, zero position, and signal damping.

**Performance**

**Optical parameters**

During early design stages of the aggregometer it was determined visually that, in agreement with theory, larger aggregates scatter light through a much larger angle than do single cells. A cuvette containing a partially aggregated suspension of cells was positioned in a beam of light focused upon a small beam stop which absorbed all
of the unscattered light. The sample was then observed at a series of angles (θ, see Fig. 2) ranging from 0° (directly behind the beam-stop) to 16°. At small θ the suspension appeared uniformly luminous. As the angle of observation (θ) increased, the uniform glow of the suspension dimmed, and the larger aggregates emerged as distinct bright particles (Fig. 4). In agreement with this observation, the combination of small beam-stop and large tube mask, which permits measurement of light scattered at small θ (3–5°), maximized the decrease in the photomultiplier tube signal as single cells disappeared (Golub, 1977). By monitoring the intensity of the unscattered, focused beam of light, it was determined that the decrease in small-angle light-scattering as cells aggregate significantly exceeds (by about 50%) the concomitant increase in transmittance (Fig. 5). This was evident both in the initial slope of the ‘aggregation curve’ (rate of decrease in printed voltage with cell aggregation) and in the curve’s total vertical excursion. This optimal combination of small beam-stop and large tube mask was used in all further experiments.

Sensitivity to aggregation showed almost no wavelength dependence. A blue filter was used routinely to match the photomultiplier tube’s sensitivity range (Vanous, 1978).

Mechanical parameters

Preliminary aggregation trials with chick embryonic neural retina cells established 15–60 rev/min as the useful range for operation of the cuvette wheel (Golub, 1977). The ‘best’ aggregation was produced at 32 rev/min with a stirring bubble of approximately 0.08 ml. At slower speeds, a small number of large cell clumps appeared, but changes in light-scattering were slow. At faster speeds, the initial slopes were steeper, but only very small aggregates (in great numbers) were formed. These results agree with those of Morris (1976), who found an inverse relation between aggregation rate and size of 24-h aggregates.

Very small bubbles (< 0.03 ml) gave poor aggregation, and elimination of the bubble prevented aggregation almost completely. Variation in bubble size from 0.05 to 0.18 ml had little effect upon aggregation. Cuvette volume, varied independently of bubble size by adjusting the thickness of the cuvette by ± about 10%, also had negligible effect. Nevertheless, care was taken to keep cuvettes uniform, and all tests were run using 0.55 ml as a standard sample volume (0.08 ml bubble).

At 32 rev/min up to 384 independent measurements – 32 per channel – are made each minute. With the print/advance command unit set to operate every 2 revolutions of the cuvette wheel, a point is recorded every 3.75 s, and consecutive points on a given curve are printed at 45-s intervals. Each such point represents the sum (average) of 24 successive measurements, and the dispersion in the data is therefore almost negligible. Assays are typically run for 30–120 min, but initial slopes of aggregation curves can be accurately determined within 10 min.

Linearity of response

The circuitry was tested for linear response to input by substituting for the photomultiplier tube signal an adjustable d.c. voltage from a constant current/voltage source. In the absence of a signal, all channels were adjusted to zero, and with the application of a selected voltage, each channel printed at a fixed voltage with no drift.
Fig. 4. An aggregated cell suspension observed by scattered light at 4 angles from the axis of illumination. All 4 photographs were taken of the identical undisturbed sample in the course of 1 min. A, $0^\circ$; B, $5.5^\circ$; C, $11^\circ$; D, $16^\circ$. $\times 5.4$. 
A 12-channel recording cell aggregometer
The channels were then equalized at this voltage using the time-constant regulators. For capacitor charges below 0.5 V, the machine's response after this initial calibration was linear within 4% for all channels (deviation from the mean of the ratios of applied voltage to printed voltage); and for capacitor charges below 0.2 V, the maximum deviation was 2% for any single channel. Printed voltages were therefore held below 0.2 V for all experimental work.

This series of tests was then repeated using the photomultiplier tube signal. With the beam stop in place, the photomultiplier tube, operated at 1500 V, emitted a strong signal with a ground glass in the beam path and no signal in its absence. This signal was used in re-equalizing the channels with the time-constant regulators to adjust for small, channel-specific, optical differences. Response (relative lamp intensity v. printed voltage) was linear within 2% except at very low and very high lamp intensities. The lamp was subsequently maintained at a given color temperature, and the beam intensity was varied as desired with neutral density filters.

Fig. 5. Aggregation of $7 \times 10^4$ neural retina cells/ml expressed as 'percent of the maximum decrease in light transmittance' (•) (increase in transmittance) (○), and 'percent of the maximum light-scattering' (decrease in light-scattering) (●). Scattering readings were made using a beam-stop 7 mm in diameter and an annular tube mask of 13 mm inside diameter. Transmittance readings were made using a tube mask with a central 2-mm aperture permitting only the focused, unscattered light to enter the photomultiplier tube. No beam stop was used, and the light intensity was adjusted with neutral density filters so that the maximum printed voltage during transmittance tests was equivalent to the maximum printed voltage obtained during light-scattering tests. Each curve represents the average of 6 duplicates run simultaneously.

* Maximum transmittance is that obtained with no cell sample in the beam path. A decrease in transmittance is produced by placing a cell suspension in the beam path. Maximum decrease in transmittance is that produced by an aggregating cell suspension at $t = 0$.

Reproducibility

All channels were equalized to 154 ± 1 mV (mean printed voltage ± standard deviation), and a standard aggregation assay was performed using 8 uniformly prepared cuvettes loaded with identical volumes of the same cell suspension. The resulting plots are reproduced in Fig. 6. The mean initial voltage was 159 ± 2 mV and the mean voltage after 60 min was 89 ± 3 mV. The mean slope for the first 10 min was 3.14 ± 0.08 mV/min.
A 12-channel recording cell aggregometer

Fig. 6. Test of assay reproducibility. A strip chart record of neural retina cell aggregation in 8 uniformly prepared cuvettes loaded with identical samples and assayed under standard conditions.

Effects of particle concentration

For a monodisperse suspension of latex beads (polystyrene; Polysciences) with a mean diameter of $5.7 \pm 1.5 \mu m$ (standard deviation), printed voltage was linear with respect to concentration from the lowest concentration tested ($1 \times 10^6$ beads/ml) up to approximately $7.5 \times 10^6$ beads/ml (Fig. 7). For a suspension of dissociated 7-day
chick embryonic neural retina cells (4–7 μm diameter), both the initial voltage and the initial slope of the aggregation curve were linear with respect to cell concentration from approximately $1.25 \times 10^6$ to approximately $7.5 \times 10^6$ cells/ml. Above these values equal increments in particle concentration produced progressively smaller increments in both parameters (Figs. 7–9). No aggregation was detectable at concentrations below

![Fig. 7. Plot of voltage as a function of polystyrene latex bead concentration (5-7 μm diam.).](image)

![Fig. 8. Plot of initial voltage as a function of neural retina cell concentration.](image)

![Fig. 9. Plot of initial slope (average slope for the first 10 min of aggregation) as a function of neural retina cell concentration.](image)
A 12-channel recording cell aggregometer

$1 \times 10^6$ cells/ml (see Fig. 12, p. 14). Based upon these results, $5 \times 10^6$ cells/ml was chosen as the standard retinal cell concentration for our experiments.

Particle size dependence of response

In order to determine the contributions of particles of various size classes to the light-scattering measured at various times during an aggregation assay, monodisperse suspensions of latex or glass beads ranging in size from $5.5$ to $90.7 \, \mu m$ (mean diameter) were prepared and tested for their light-scattering at small $\theta$. The $5.5-\mu m$ glass beads and the $5.7-\mu m$ latex beads, roughly equal in diameter to a chick embryonic neural retina cell, were used at a concentration of $5 \times 10^6$ beads/ml, while the larger beads were used at equal partial volumes (lower concentrations). Measurements made using these suspensions reveal, as expected, that small-angle light-scattering by a given volume of beads decreases as the mean diameter of the beads increases (Fig. 10).

With the angular settings adopted for these experiments, the aggregometer is very sensitive to changes in the size of particles (at constant partial volume) smaller than about $12 \, \mu m$ and very insensitive to changes in the size of particles larger than about $25 \, \mu m$.

![Graph](image)

**Fig. 10.** Plot of per cent maximum light-scattering as a function of bead diameter. A constant partial volume equivalent to that of $5.7-\mu m$ diameter latex beads at $6 \times 10^6$ beads/ml was maintained by decreasing the bead concentration as bead diameter increased. Data for latex beads (●) were prepared using the readings for $5.7-\mu m$ latex beads as maximum light-scattering. Data for glass beads (○) were prepared using the readings for $5.5-\mu m$ glass beads (Polysciences) as maximum light-scattering.

In a second set of experiments (Allen, 1978), the particle size distributions in suspensions of aggregating cells ($5 \times 10^6$ cells/ml) were determined by counting single cells, doublets, triplets and quadruplets-or-larger in samples taken at intervals during the assays. From these data, a standard curve was drawn of the approximate single-cell concentration at any given time in a typical aggregation assay (Fig. 11). Cell suspensions ranging in concentration from 1 to $5 \times 10^6$ cells/ml were then prepared and assayed for their initial aggregation (Fig. 12). Each of these curves was compared
with the corresponding segment (representing the same single-cell concentration) of a typical aggregation curve in order to estimate the contribution of single-cell aggregation to the total aggregation measured at each time (Fig. 13). Consideration of particle distribution data (Tables 1 and 2) made it possible to estimate the contributions of other particle size classes to the measured aggregation.

The decrease in single cells accounted for almost all measured aggregation initially, but its relative contribution diminished considerably through the course of the assay.

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Fig. 11. Single-cell concentration as a function of time in an aggregating cell suspension. Values were determined by haemocytometer counts of samples taken from individual cuvettes. Each point represents the mean ± standard deviation of values from 4 tests.

Fig. 12. Aggregation of neural retinal cell suspensions at different cell concentrations:
- $1 \times 10^5$ cells/ml,
- $2 \times 10^5$ cells/ml,
- $3 \times 10^5$ cells/ml,
- $4 \times 10^5$ cells/ml,
- $5 \times 10^5$ cells/ml.
Fig. 13. The contribution of aggregation of single cells to the total measured aggregation. The average initial slope of each plot from Fig. 10 is compared with the slope of that portion of a typical aggregation curve with an equal concentration of remaining single cells (as determined from Fig. 12).

Table 1. The number of aggregates in each of 4 size classes (single cells, doublets, triplets and quadruplets or larger) as determined by haemocytometer counts of samples taken after 0, 5, 15, 30 and 60 min of neural retinal cell aggregation

<table>
<thead>
<tr>
<th>Min of aggregation</th>
<th>Aggregate size class</th>
<th>Total no. of aggregates counted/sample</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>0</td>
<td>885</td>
<td>121</td>
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<tr>
<td>5</td>
<td>875</td>
<td>137</td>
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<td>15</td>
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<td>171</td>
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<td>30</td>
<td>784</td>
<td>178</td>
</tr>
<tr>
<td>60</td>
<td>715</td>
<td>151</td>
</tr>
</tbody>
</table>

Table 2. The percentages of total aggregates contained in each of the 4 size classes after 0, 5, 15, 30, and 60 min of aggregation

<table>
<thead>
<tr>
<th>Min of aggregation</th>
<th>Aggregate size class</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>0</td>
<td>84 %</td>
</tr>
<tr>
<td>5</td>
<td>80 %</td>
</tr>
<tr>
<td>15</td>
<td>76 %</td>
</tr>
<tr>
<td>30</td>
<td>71 %</td>
</tr>
<tr>
<td>60</td>
<td>66 %</td>
</tr>
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The contribution of small aggregates to the light-scattering through small $\theta$ is inversely related to their mean diameter. Since they appeared early and remained numerous at all subsequent times, their coalescence contributed significantly to the total aggregation measured after the first few minutes. Large aggregates became increasingly large and numerous as aggregation proceeded. They themselves contribute little to the measured light-scattering but probably functioned as collecting aggregates to help remove remaining single cells and small aggregates.

**DISCUSSION**

The particular utility of our instrument for measuring aggregation or agglutination is due largely to 3 features: (1) the monitoring of small-angle light-scattering, (2) the cuvette design, and (3) the data processing.

The progression of events described above for a typical aggregation has been established through particle counts (Steinberg et al. 1973; Fig. 11 and Tables 1 and 2) and through direct observation of aggregating suspensions. Some procedures for assaying cell aggregation have monitored a single class of events such as the disappearance of single cells or the appearance of larger aggregates. By contrast, monitoring changes in small-angle light-scattering makes our aggregometer sensitive to several classes of adhesion events that occur to different degrees throughout the course of cell aggregation.

Our cuvette differs in several respects from the bonded glass cuvette used by Beug & Gerisch (1972). It is durable and, due to the ease of its dismantling and reassembly, rapidly cleaned. The optical surfaces (standard glass coverslips) are replaced after each use. In our experience, the discoid chamber has also proved to be better suited for the study of chick neural retina cell aggregation than the racetrack-shaped chamber of the Beug-Gerisch cuvette. We observed in comparison tests that, as the long axis of the latter cuvette passes through horizontal, the stirring bubble races from one end of the cuvette up the straight side to the other end, creating a high transitory shear which we found to be largely rev/min-independent and severely inhibitory of retina cell aggregation. The stirring in the discoid chamber of our cuvette is constant, gentle and a direct and continuous function of rev/min.

With our data processing system, measurements made in each cuvette every 1.88 s are automatically sorted out, summed (averaged) and printed every 45 s. Up to 12 separate aggregation curves comprising a total of 23040 independent measurements (1920/cuvette) can thus be condensed into 960 printed points (80/curve) per h. The system is reliable and permits many aggregation or agglutination assays to be conducted simultaneously and rapidly.

We are grateful to Drs G. Gerisch and H. Beug for providing details of the construction of their aggregometer; to Russell Mycock, Mario Lavolva, and Dr George T. Reynolds for advice and assistance with mechanical, electronic and optical designs; and to Robert M. Golub and Ray M. Allen, Jr., undergraduate students who participated in the initial testing of our instrument.

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