

Mixing in 384-Well Plates: Issues, Measurements, and Solutions

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Abstract: Mixing in standard 384-well plates is different from mixing in 96-well formats. The aspect ratio of a typical well, the balance of surface tension and mass of the fluids, and the scale of diffusion all add to the increased difficulty in mixing fluids in higher-density plates. Here we examine two methods to measure mixing and some common techniques for mixing in 384-well plates. While conventional shaking can suffice, alternative methods can accelerate and improve the efficiency of mixing in 384-well plates.

Introduction

THE SOCIETY FOR BIOMOLECULAR SCREENING standard 384-well plate format is prevalent in HTS groups, compound library management groups, and discovery areas. Well sizes change in diameter from approximately 8 mm in a 96-well plates to 3.5 mm in 384-well plates. The physical phenomena and mathematical scaling related to this change of length scale point to potential problems for mixing of fluids. Exploration of these problems led to development of solutions and methods to quantitate mixing. These findings apply to compound formatting and discovery research through follow-up assays. Insights into the quality and appropriateness of mixing methods through dye testing may enable scientists to better understand and control experimental variability.

Scaling an assay down from 96- to 384-well plates is not straightforward. There is a tendency to reduce the working volume by about half. However, the tall, narrow wells of 384-well plates have a two to three times greater aspect ratio than the wells in 96-well plates. The length scale is reduced by half, the area is decreased by 4, and if one wants to keep the same aspect ratio, volume should be decreased by a factor of 8. The well is too large for diffusion alone to mix fluids, even within hours.¹ The effects of viscosity, surface tension, and gravitational forces change disproportionately from 96- to 384-well

format. Complete mixing in 384-well plates is difficult to obtain as compared with lower-density plate formats.²

The ideal method for mixing would produce the maximum amount of interfacial area between two initially segregated fluids in the minimum amount of time or using the least amount of energy.³ Mixing is crucial for data reproducibility between wells and within wells. The homogeneity of an assay solution will have profound effects on the results of that assay, whether it is a straightforward biochemical or more complex functional assay.

There are three major mechanisms of mixing: diffusion, dispersion, and convection. Diffusion of aqueous reagents is limited because of a narrow experimental temperature range. Techniques exploiting dispersion and gas sparging mix effectively,⁴ but are not well suited for 384-well plates where scattering is undesirable between wells and gas introduction within the wells is impractical. Convection mechanisms are the most widely used and readily modified for 384-well plates. There are four categories of convection available in the typical biochemical lab: orifice-jetted addition, pipetting, stirring, and shaking.

Orifice-jetted liquid handling devices are generally offered as non-contact bulk dispensers. These dispensers typically use an eight- or 16-channel manifold to distribute reagents across a plate. A sufficient velocity at the tip is required to break off the fluid from the manifold orifice. This velocity may cause fluid turnover in the well,

Amgen Inc., Thousand Oaks, CA.

ABBREVIATIONS: CV, coefficient of variation; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; RFU, relative fluorescence units; SD, standard deviation.

which is the goal of mixing. Bulk dispensers can be placed in-line in assay processes, and may enable efficient mixing. Drawbacks include unwanted violent flow in the well, especially troublesome with adherent cellular assays, where shear stress on the cells is best avoided.

Pipetting (contact aspirate, contact dispense) is a much more gentle addition to the well, and therefore may not mix well. A common practice to achieve mixing during plate preparation is to aspirate buffer, an air gap, followed by the compound of interest into the tip and to dispense the entire volume into the well. Similar to hand pipetting, a few cycles of aspirating from the bottom and dispensing at the top of the well can be an effective form of mixing.

Efficient mixing can be accomplished by stirring. Several variations are possible in which 384-well plates can be mixed within seconds. Useful methods include the use of disposable pin tools. Pin tool arrays are used to stir the plate either by orbital shaking of the plate allowing a loose pin tool array to oscillate in the wells, or by shaking the pin tool array relative to the plate. A variation is to use a 384-pipettor head with its tips extended into the plate while the plate moves on an orbital shaker.

Orbital shakers are perhaps the most common post-process mixing devices. The plate is orbited in a tight circle, and wall contact causes shear as the fluid tries to follow the orbit. Most shakers are designed for 96-well plates and are not optimized for higher-density plates with smaller wells.

Each method has its applications and drawbacks in biochemical assay processes. The assay itself may require different degrees of mixing. A moderate-rate-kinetics biochemical assay may not require stringent homogeneity to get accurate data, while a cellular assay may be highly sensitive to local reagent concentrations and therefore require significant and rapid mixing homogeneity at all points in the assay while leaving the cells unperturbed. It is not uncommon for an assay to dictate an in-line process with adequate mixing in under a minute.

Methods that quantify mixing are vague or overly complicated,⁵ and the definition of mixed may not be consistent across applications. Well dispersed does not necessarily mean well mixed. Conversely, a locally blended fluid might be stratified in its container, and should not be classified as well mixed. For example, a compound solution might fall to the bottom of a well very consistently, be mixed laterally, but actually be at a much greater concentration at the bottom of the well than intended in the assay design. We define a well-mixed state as one in which the compound is well distributed throughout the well, but is also intimately blended at the smallest measurable scale.

Detection systems may not be able to discern heterogeneities. For example, plate readers average by collection over the entire well, giving only an overall value,

possibly missing information regarding fluid variations. In some cases, camera imaging can give much greater information. The images retain data from the scale of the entire well down to the pixel resolution of the imaging system. Imaging from the side of the well will give both horizontal and vertical information.

In this paper we have examined some common mixing methods using exaggerated solution combinations to get an understanding of the degree of mixing that occurs in 384-well plates. This study will examine two different detection systems. These detection systems will then be used to compare the effectiveness of different mixing methods. Based on the results, recommendations for improving mixing in 384-well plates will be given. The aspects related directly to cellular assays are expansive and will be discussed in another paper. The authors have intentionally limited the scope of these studies to the extreme cases of dimethyl sulfoxide (DMSO) and aqueous mixtures with DMSO in order to highlight the behavior of mixing methods. Various combinations of fluids and ratios of these fluids are out of the scope of this examination.

Theory

The science of mixing is not new. Reynolds⁶ published on turbulence in the 1880s. Fick's law governing diffusion was published in 1855.⁷ Using the derivation of Fick's Law for semi-infinite volume and a diffusion constant for a small molecule in aqueous solution at room temperature, the concentration at 2.5 mm from the original interface is only 20% after 1 h.¹ Diffusion alone is limited and will not account for significant mixing on its own. DMSO is fully miscible in water⁸; however, miscibility does not necessarily correlate to kinetics. In the case of two dissimilar fluids like DMSO and water, the time it takes to come to equilibrium may be excessive.

Empirical methods have been used to study mixing phenomena for a variety of cases. Rousseaux *et al.*⁵ have recently correlated forced vortex (impeller) behavior with microscale mixing. Kato *et al.*⁹ have done extensive modeling of orbital shaking in large-scale reactors. In a critical range of frequencies, the sloshing in the well changes from progressive wave to a rotational flow. Weiss *et al.*¹⁰ have modeled orbital shaking in 96-well plates. In this report, the authors identify the importance of in-phase orbital oscillation to efficient mixing, and report on a method to quantitate flow patterns using pH-sensitive dyes.

Dimensionless numbers such as Froude, Weber, and Reynolds relate applied forces to gravity (length), surface energy (area), and viscosity (volume), respectively. There are critical values associated with each of these dimensionless numbers that indicate a behavioral change. Table 1 is a summary of the dimensionless values and

TABLE 1. SUMMARY OF DIMENSIONLESS PARAMETER VALUES

| | <i>Froude</i> | <i>Weber</i> | <i>Reynolds</i> |
|----------------------|---------------|--------------|-----------------|
| 96-well plate value | 0.3 | 0.2 | 250 |
| 384-well plate value | 0.9 | 0.4 | 250 |
| Critical value | 1.0 | 1–3,000 | 2,400 |

the corresponding critical value for noted phenomena changes. A Reynolds number of 2,400 is the theoretical value between turbulent and laminar flow; above this value is the turbulent regime, below is the laminar regime.¹¹ A Weber number larger than unity may indicate droplet formation, but this critical number varies with experimental condition.^{12–14} A Froude number near unity indicates wave breaking or gravity currents. Below unity, gravity dominates the flow behavior.^{15,16} While this report's focus is not on 96-well plate mixing, it is important to see the nonlinear nature of these dimensionless numbers as they scale from typical 96- to 384-well plate wells. Calculations used a dimension of 4 and 8 mm diameter and 1,200 and 2,400 rpm for the 96- and 384-well plates, respectively. Water was used as the fluid with 72 mN/m surface tension and 0.89 mPa/s viscosity.¹⁷

Orbital mixing involves an exchange of liquid between positions within the well. To get this exchange, the surface must first deform into a bulb, and that bulb must be moved along the wall of the well by the oscillation. However, excess agitation will cause satellite droplets to be expelled, which defeats the well's purpose. A schematic of this motion is shown in Fig. 1. The aspect ratio of height to diameter for a well in the 96-well plate format is about 1; for a well in the 384-well plate the aspect ratio is closer to 2. The taller aspect ratio well will be less efficient in the vertical exchange of fluid given the perturbation from orbital shaking, which is primarily horizontal.

While the essential physics does not change, the scale of interactions does change as 96-well assays are transferred to 384-well format. For example, surface tension does not scale with gravimetric effects of the well volume. Assuming a well 8 mm in diameter containing 150 μ l of water with surface tension of 72 mN/m, the surface tension over the circumference of the well is roughly 1.8 mN. The gravimetric force is $m \times g$, 1.4 mN. The surface tension is slightly greater than the force of gravity. This approximation is supported in laboratory experiments in which a partially filled 96-well plate can be carefully turned over without loss of fluid. Any additional acceleration from sudden movement would add to gravity to overcome the surface tension and spill. For a 384-well plate with 50 mg (50 μ l) and 4 mm in diameter, the surface tension is 0.9 mN, and the gravimetric force is 0.47

mN. This plate, when turned over, will not spill; it requires about twice the acceleration of gravity to remove liquid from the wells. This linear dependence is expected not because of the change in length scale, but because of the change in volume per area, *i.e.*, L^3/L^2 .

The coupling of oscillation frequency with fluid movement has been shown by Weiss *et al.*¹⁰ Further refinement may include momentum, surface changes, curvature, viscosity, orbital frequency, and perturbation theory, but this modeling is irrelevant if fluid exchange cannot be established.

Materials and Methods

The Safire™ (Tecan Schweiz AG, Männedorf, Switzerland) was set to bottom-read at 585 nm excitation and 610 nm emission for fluorescence-based plate reader studies. Sulforhodamine (S101) (CAS number 518-47-8, Sigma, St. Louis, MO) was mixed with DMSO (CAS number 67-68-5, Alfa Aesar, Ward Hill, MA) to make a 10 μ M dye solution. Forty microliters of deionized water was dispensed using the Multimek (Beckman Coulter, Inc., Fullerton, CA) into clear-bottom plates (clear-bottom black 384-well polystyrene, Corning, Corning, NY). Ten microliters of the dye solution was layered under the water to create the initial layered condition.

For the shaker studies, eight plates were used for each experimental time series. The TEOS (TechElan, Mountainside, NJ), Big Bear Automation shaker (Big Bear Au-

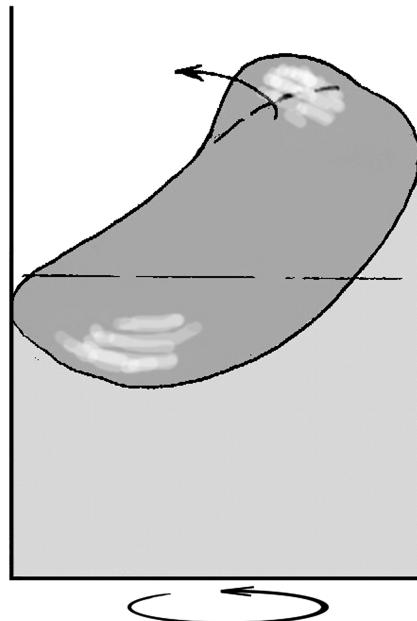


FIG. 1. Schematic of the desirable motion from orbital shaking in order to exchange fluid within a well.

tomation Inc., Pleasanton, CA), VarioMag® shaker (H+P Labortechnik AG, Oberschleissheim, Germany), and the Belco orbital shaker (Belco Glass, Vineland, NJ) were used to mix the plates. In stirring experiments, either loose pin tools (P/N XGE5050, Genetix, New Milton, Hampshire, UK) were shaken in the plate, or pipette tips were left submerged on the MiniTrak™ (Perkin Elmer, Wellesley, MA) while the Variomag shaker was active.

For the pipetting series, seven plates were used to analyze aspirate–dispense cycle series. The 384-head Multimek used P30 disposable tips with an orifice diameter of 0.38 mm (0.015 in.) (P/N 82007-296, Labcon, Petaluma, CA), and syringe speeds were run at 50% maximum.

An Itronics Inc. (Westlake Village, CA) FastCam PCI 1280 high-speed camera was set to collect 1,000 frames per second for imaging studies. India ink (Pelikan drawing ink number 17, black) was mixed at 0.75 ml per 100 ml of DMSO to make 100% dye solution or 0.75 ml of ink per 100 ml of 10% DMSO and 90% phosphate-buffered saline (PBS) (1×, Gibco, Grand Island, NY) to make 10% dye solution. For the pipetting and shaking studies, 40 μ l of PBS was dispensed using the Multimek into clear polystyrene plates (clear flat-bottom 384-well polystyrene, Corning). Ten microliters of dye solution was layered under the PBS to make the initial layered plates.

On the Belco shaker with pin tools, both methods used 100% dye solutions layered under deionized water.

When the in-house design of the bulk dispenser was used, 10 μ l of ink solution was layered under 20 μ l of PBS to make the initial layered plate, and 20 μ l of clear PBS was dispensed to image the mixing turnover.

For the pipetting series, images were collected continuously as cycles were run either on the Multimek as described above or on the Vprep. The Vprep used a fixed-tip head with a diameter orifice of 0.22 mm (0.0085 in.) (Velocity 11, Menlo Park, CA), and speeds were set to 90 μ l/min.

For the shaking series, images were collected for each shaking time in the method comparison on the Belco with pin tools. Images were collected continuously for the shaking study using the in-house design shaker.

Image analysis was done using a custom application in MatLab (MathWorks, Natick, MA) that displayed regions of interest and calculated statistics.

Quantitation methods

For this study, a fast and easy quantitative method was desired. Two methods to quantify mixing are introduced: one using fluorescent dye for plate reading, and one using India ink for high-speed camera imaging studies. In both cases, the wells are carefully loaded with the dye solution at the bottom and clear fluid

above it. If mixing is efficient, imaging will show the distribution, and the fluorescence reader will see a decrease in photon count.

Plate reading method

A model of quiescently loaded dye in buffer is shown in Fig. 2A. The Safire focuses on a region near the bottom of the well when set to detect epifluorescence from the bottom of a plate. This feature can be used to examine the relative amount of fluorophore at the bottom of the well. If we have efficient exchange in our test wells of fluid from top to bottom, the relative fluorescence units (RFU) counts will decrease from the initial, highly concentrated layer as shown in Fig. 2B. While an absolute quantitation is not required for this study, the RFU counts can distinguish between mixed and unmixed states. This is especially relevant in the case of orbital shaking, where the primary motion is horizontal. A dilution of the dye at the bottom of the well will signify efficient mixing.

To establish values for the mixed condition, the value after thorough mixing with pin tools is used as a baseline. This method proved to be the most efficient and well correlated to the experimental data, and will be examined in Results. A mixed condition is defined as within 1 standard deviation (SD) of the baseline values.

The test procedure was as follows: fill Corning Costar 3711 plates with 50 μ l of deionized H₂O using the 384-head Multimek. Aspirate 20 μ l of 0.01 mM S101 in DMSO and dispense 10 μ l at 2.5% speed near the bottom of well. Spin the plate at 15,00 rpm for 5 min and then allow the plate to sit 10 min. Mix the plate per test

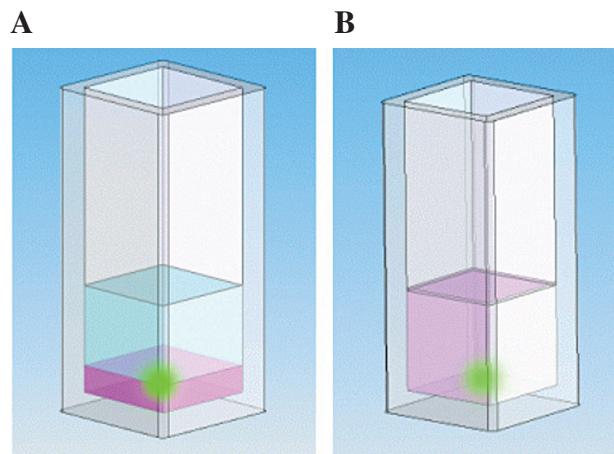


FIG. 2. (A and B) Schematic of a single well of a 384-well plate containing 50 μ l. Using the plate reader's focused bottom reading capability (shown in green), fluorescence intensity allows for quantitation of the degree of mixing. Unmixed wells will have a relatively high photon count; mixed wells will show a decreased count.

conditions. Read on the Safire (bottom-read with 585 nm excitation and 610 nm emission).

Commercial devices were tested with plates prepared as above. Eight plates per device were measured, one after each mixing time of 0, 5, 10, 20, 30, 40, 50, and 60 min.

Imaging method

The high-speed camera (Itronics FastCam PCI 1280) was set up to image the side of a clear polystyrene 384-

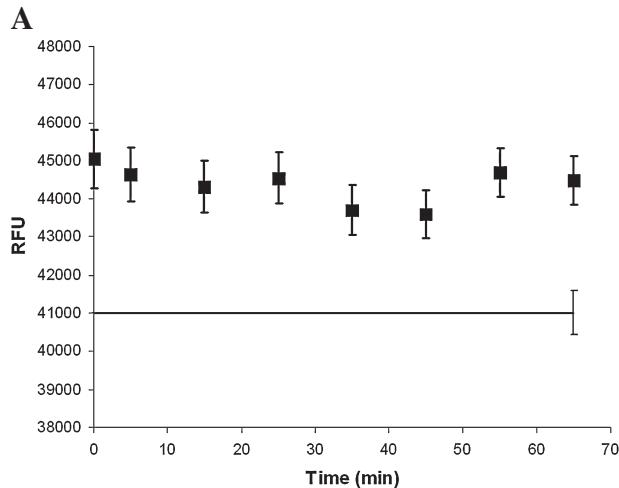


FIG. 3. (A) Graph of plate reader fluorescence counts of concentrated dye at the bottom of a well over time, for untouched (■) and the mixed reference line (—). The initial setup had 0.01 mM S101 in 100% DMSO, layered below water. Error bars represent the SD of $n = 384$ values. (B) Graph of fluorescence counts of dye at the bottom of a well over the number of pipette mix cycles (◆) and the mixed reference line (—). For the mixing, dispensing was done approximately 10 mm from the bottom, and aspiration was done approximately 2 mm above the bottom of the well. The initial setup had 0.01 mM S101 in 100% DMSO, layered below water. Dispense was set to half-maximum speed. Error bars represent the SD of $n = 384$ values.

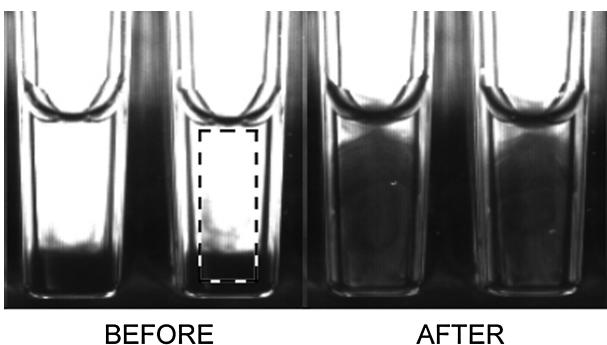


FIG. 4. Before and after images of state of wells for five modified aspirate-dispense cycles on the Multimek. Initially 40 μ l of 1× PBS was in the well before 10 μ l of ink solution was added. For the mixing, dispensing was done approximately 10 mm from the bottom, and aspiration was done approximately 2 mm above the bottom of the well. Dispense was set to half-maximum speed.

well plate. The plate was prepared by removal of the side skirt and by painting the second row of wells, behind the wells to be imaged, to reduce reflections. Plates were filled with layered fluids in the same manner as described above, with the following differences per experiment. For the bulk dispense, plates had 10 μ l of ink solution under 20 μ l of PBS. For all other tests, 10 μ l of ink solution was layered under 40 μ l of PBS. For the bulk dispense, an additional 20 μ l of PBS was dispensed into the wells during imaging. For the syringe-based tests, tips drew from the existing wells. For the shaking and stirring tests, plates were used as prepared.

Image analysis was done in order to quantify the extent of mixing in the collected images. An initial condi-

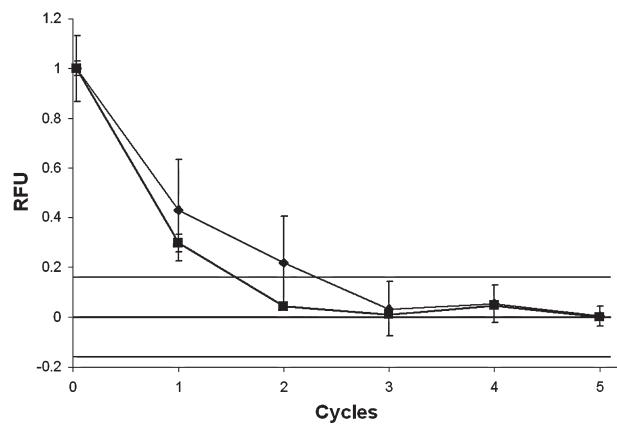


FIG. 5. Normalized comparison of plate reader RFU data (◆) with image analysis pixel CV data (■). Normalization scaled the maximum value of each method as 1.0 and the minimum value as 0.0. The center line is the average mixed value of a plate, with lines representing 1 SD above and below. Error bars for the fluorescence data are normalized SD of $n = 384$ values; error bars for the pixel analysis are $n = 3$ image averages.

tion of black ink layered under clear fluid against a white background allows pixel values to indicate mixing homogeneity. Absolute grayscale values were not appropriate for comparison because of differences in imaging conditions. The coefficient of variation (CV) of pixel values in a single well was useful to describe the homogeneity of the fluids in the wells. CV calculation assumes a normal distribution to get a legitimate absolute value; while unmixed image grayscale values were bimodal and not distributed normally, the relative differences in CVs between mixed and unmixed wells was useful for rough quantitation. A minimum of 80 pixels top to bottom and no less than 10 pixels across were collected, depending on image conditions. Three wells per plate were averaged in the reported CV_{well average}.

Results and Discussion

As wells become smaller, requirements for physical movement of the fluid are different. The physics may be the same, but the scales of the physical interactions are

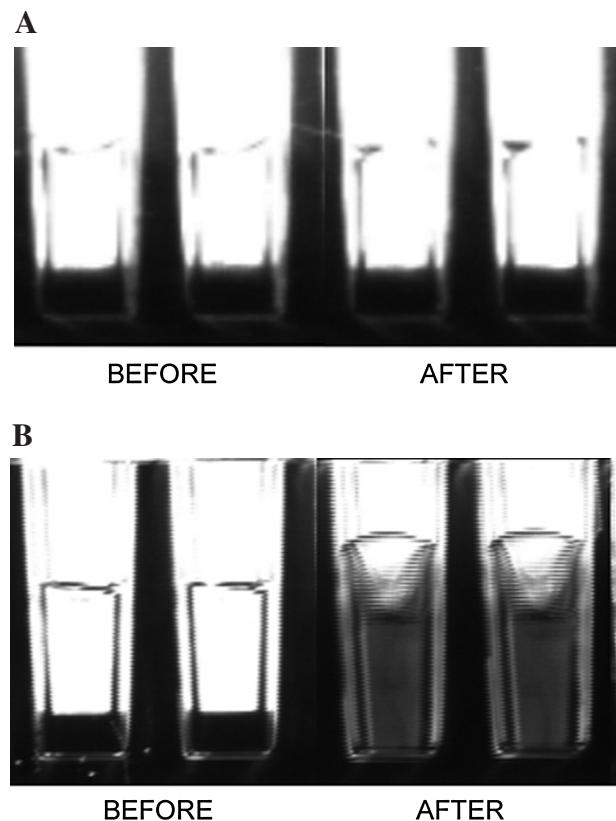


FIG. 6. Images of before and after shaking for 5 s: (A) 100% and (B) 10% DMSO solution. Forty microliters of 1× PBS was layered above 10 μ l of each ink solution. The plates were shaken for 5 s at approximately 60 Hz on the in-house shaker.

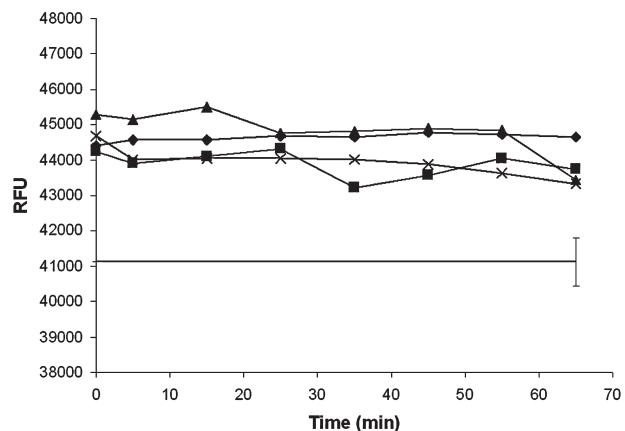


FIG. 7. Graph of fluorescence counts reflecting dilution from mixing of concentrated dye with solvent using Belco (◆), Big Bear (□), Tech Elan (▲), and VarioMag (×) shakers. The initial setup had 0.01 mM S101 in 100% DMSO, layered below water. Speed settings were “6,” maximum, maximum, and 384-maximum, respectively. Error bars represent the SD of $n = 384$ values. SDs for all plates measured were between 508 and 783 counts, roughly equal to the error bars shown on the mixed line (—). For image clarity, error bars are not shown.

not. First, the two quantitation methods are compared. With these methods, shaking, stirring, pipetting, and bulk dispensing techniques are examined.

Quantitation comparisons

Two quantitation methods were used to examine the degree of mixing in 384-well plates. The fluorescence reader method indicates fluid exchange from the bottom of the

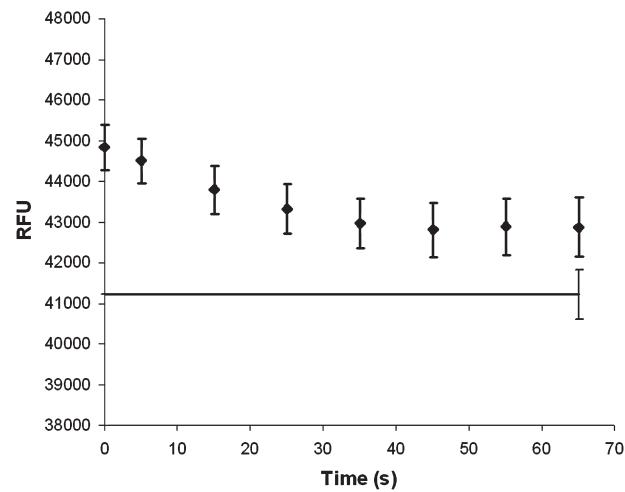


FIG. 8. Graph of fluorescence counts reflecting mixing using the pin tool suspended in the wells while shaking on the Belco shaker (◆). Note the time scale on the x-axis. The initial setup had 0.01 mM S101 in 100% DMSO, layered below water. Each point in time is a separate 384-well plate. Error bars represent the SD of $n = 384$ values.

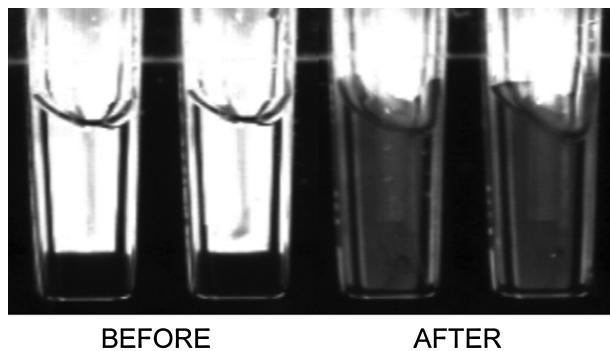


FIG. 9. Images of pin tool mixing before and after 2 s of shaking. Initially 40 μ l of 1 \times PBS was layered above 10 μ l of the 10% DMSO ink solution. The pin tool was shaken at approximately 60 Hz.

well. Imaging not only provided qualitative understanding, but could also be quantified through pixel statistics. The unmixed state was created by careful layering of dye below clear buffer or water. The mixed state was created by actively folding the two fluids together with the Multimek.

Photobleaching was checked and discounted through separate large-volume experiments (data not shown).

Figure 3A shows the difference between mixed and unmixed states by fluorescence measurements. In the unmixed state, the fluorophore is concentrated at the bottom of the well and, as it is mixed, dilutes with fluid above. The data show that diffusion alone is not a significant enough to mix the fluids in an hour. Plate-to-plate variation fell within a single SD of a given plate's statistics, and the average counts for the unmixed state are significantly above that of the mixed plates. Figure 3B shows one robust method for fluid turnover. The fluid in the well was aspirated from near the bottom of the well and redispensed just above the top surface of the liquid. The redistribution from top to bottom of the well helps mix the two fluids.

Figure 4 shows the difference between mixed and unmixed states through image acquisition: the two wells at the left (before) are in the unmixed state; the two wells at the right of the spliced image are after five aspirate-dispense cycles. A typical area selected for pixel statistics is shown by the dashed line. This mixing method leaves a small portion of unmixed fluid at the top edges

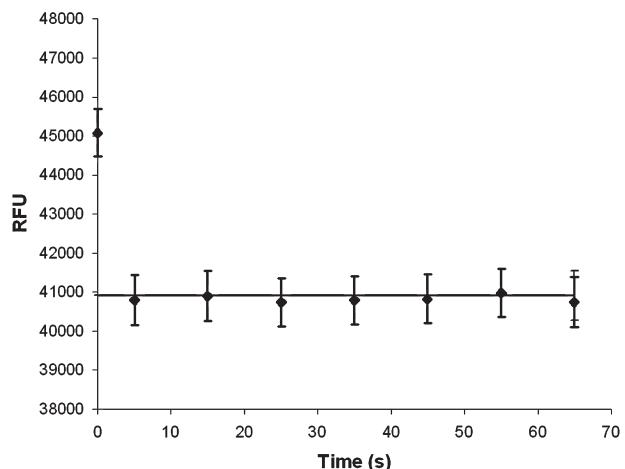


FIG. 10. Graph of fluorescence counts reflecting mixing over time. This method used disposable tips on the MiniTrak submerged in the wells while being shaken with the VarioMag shaker (◆). The initial setup had 0.01 mM S101 in 100% DMSO, layered below water. Error bars represent the SD of $n = 384$ values.

of the well. In order to quantify the images, the CV, not the average, proved useful. The average would give similar values whether mixed or not, but the CV of the pixel values represents the degree of spread between the darkest and lightest pixel values. In the case of unmixed, the CV is high, 48%, because there is a bimodal distribution of pixel values near the extremes of black and white. As the solution mixes, the CV drops to 16% because the majority of the pixel values are near the average gray, in this case, 193 out of 255. These values will change with image collection settings, with selected area, and with the manipulations necessary to quantitate the images, so reference images of the stratified and well-mixed states are needed for quantitation.

The differences between the two methods are worth noting. The imaging method views the entire well from one side and inherently averages, per pixel, along the axis of view. The plate reader is focused on the bottom volume of the well and does not examine the state of the fluids at the top of the well. It will be useful to compare the results of the two methods. Figure 5 shows normalized results for an equivalent system (ink or fluorophore)

TABLE 2. COMPARISON OF MIXING TIMES

| Shaker, method | Mixing time to within 1 SD of baseline |
|---|--|
| No mixing | >24 h |
| Belco, Big Bear, VarioMag, Tech Elan | >60 min |
| Shaker with pin tool | <5 s |
| MiniTrak and VarioMag | <5 s |
| Multimek with variable height technique | 20 s (2 cycles) |

after mixing by multiple aspirate-dispense cycles. The graph is normalized to unity for the comparison, and reveals similar behavior as the wells change from unmixed to mixed. Parameters for both methods will change the data, most notably the gain settings on a plate reader and contrast levels in an image. While these are significant differences, the normalized, relative measurements show agreement in the distinction between mixed and unmixed states.

Solution types make a difference

The images in Fig. 6 show before and after states for 10% and 100% DMSO/dye solutions. The 100% DMSO ink solution is not as easily mixed as the 90:10 water:DMSO. While DMSO is fully miscible in water, the ability to mix, given the same well geometry and shaking parameters, can change from seconds to hours by changing the amount of a solvent.

Shaking

Often, mixing requirements are simple, and standard plate shakers are employed. The shakers seem to have been designed for 96-well plate or lower-density plates because they do not reach the necessary frequency or amplitude for higher-density plates.

Four commercially available shakers were tested. In Fig. 7, the Belco, Big Bear, Tech Elan, and Variomag shakers were tested with the 100% DMSO solution. In all cases the shaking did not deviate from the unmixed state until after 20 min. The Belco, Bear, and Variomag shakers have a maximum setting that gets to approximately 1,200 rpm. This orbital frequency is apparently not enough to couple the energy needed to cause significant vertical fluid translation in the well. The Tech Elan shaker has a higher frequency capacity, approximately 4,000 rpm. While these devices do show mixing behavior, 20 min would usually be too long to wait for the first indication of mixing.

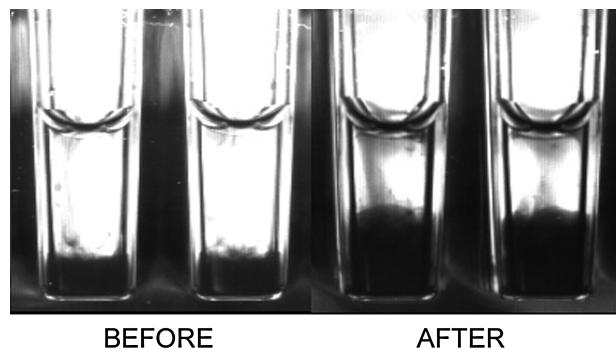


FIG. 11. Before and after images of the state of wells after five aspirate-dispense cycles on the Multimek. PBS (1×) was layered above the ink solution. Aspiration and dispensing were done approximately 2 mm above the bottom of the well.

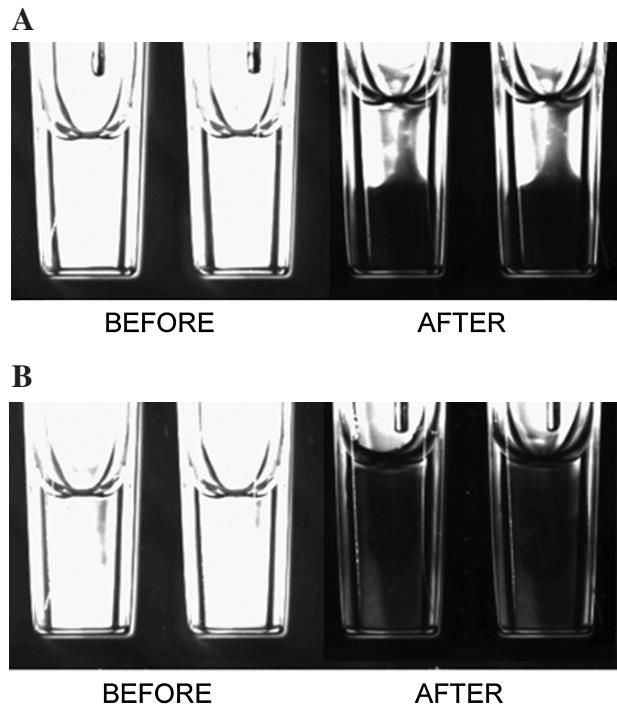


FIG. 12. (A) Before and after images of the state of wells for dispensing 10 μl of ink solution with the Vprep. Initially 40 μl of 1× PBS was in the well. Aspiration and dispensing were done near the bottom of the well. Dispense speed was 90 $\mu\text{l/s}$. (B) Before and after images of the state of wells for dispensing 10 μl of ink solution from the fixed tip of the Vprep. Initially 40 μl of 1× PBS was in the well. Aspiration and dispensing were done at the top of the fluid volume. Dispense speed was 90 $\mu\text{l/s}$.

Stirring

Figure 8 shows the amount of mixing using the shaker with disposable pin tools. The pins float freely above the plate and act to dramatically increase the convection and folding of the two fluids. Mixing is stabilized within

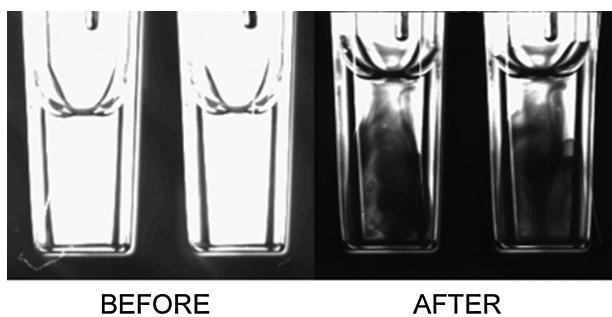


FIG. 13. Images of the state of wells before and after dispensing (no mixing) 10 μl of ink solution with the Vprep at the top of the well. Initially 40 μl of 1× PBS was in the well. Dispense speed was 90 $\mu\text{l/s}$.

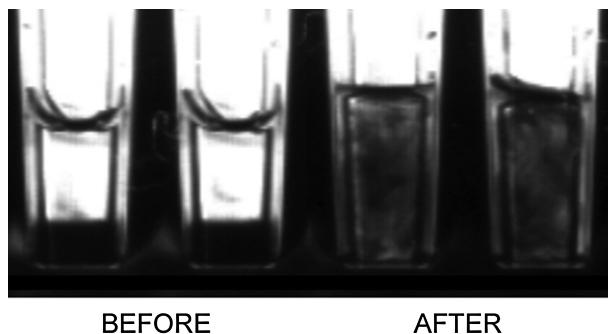


FIG. 14. Before and after images of a single dispense from a pressurized, bulk addition manifold. Twenty microliters of 1× PBS was layered above 10 μl of the ink solution. Twenty microliters of PBS was dispensed to make a total of 50 μl per well. Orifice diameter was 0.046 mm; source pressure was set to 5 mPa.

30 s; however, the final state of the experiment is not fully mixed. The pins do not touch the bottom of the well and therefore will not displace adherent cells on the well bottom. A stagnant area below the pins has been seen in pin mixing images and is reflected in the plateau above the “mixed” state values. These data attest to the precision of this method of quantitation; the focused read distinguishes a state between unmixed and fully mixed. As stated earlier, the 100% DMSO dye solution is much more difficult to mix than the 10% solution.

Figure 9 shows mixing after 2 s of shaking with a disposable pin tool held loosely in the plate. It should be noted that this is very efficient. Even shaking for 0.7 s at 60 Hz is sufficient to mix to visible homogeneity. The combination of moderate orbital speeds and the pin tool acting as a lid removed the potential for splashing between wells. Modification of the assay process (lidding and delidding) can make the addition of pin tools a viable option for an assay process. However, no commercially available pin tool will function as a lid.

The stirring method can transfer to pipetting systems. A shaker, mounted on the deck, can be turned on while the tips are in the wells of the plate. Figure 10 is an image of this method applied to the MiniTrak with the VarioMag shaker on the deck. The disposable tips were used to add solution, and then left submerged and in a fixed position as the shaker was activated. This method has very high mixing efficiency when the tips were set to be near, but not touch, the bottom.

This method has added benefits. It can be implemented within the assay process, it will add little to the cycle time, and it does not require additional consumables.

Comparisons of time-to-mix are given in Table 2. These times are for the worst-case situation of water layered over a 100% DMSO dye solution. In most cases, orbital shaking and the implied convection are insufficient to mix within typical assay timeframes. When a physical barrier is added, shaking becomes stirring, and mixing efficiency goes up.

Pipetting

Imaging acquisition is useful for in-process mixing studies, *i.e.*, where plates cannot be removed. Images of the side of the well allow for both qualitative understanding of the state of the wells at a given time, and also allow quantitation through image pixel analysis.

Figure 11 shows the side of a clear 384-well plate filled with 50 μl of water and 20 μl of India ink. The Multimek was cycled (dispense and aspirate) five times with the tips held near the bottom of the well only, with no vertical movement. The two fluids were still separated, with little exchange, and the majority of the redistribution came with the final removal of the tips. This result was consistent with a Reynolds number that was well below that for turbulent flow. The streaming of the fluids in and out of the pipette tips stays segregated. Indeed, this device has such poor mixing properties that it was used to establish the initial layered, non-mixed condition.

TABLE 3. COMPARISON OF SOLUTION HOMOGENEITY BY IMAGE ANALYSIS

| Method/device | $CV_{\text{well average}}$ | |
|---|----------------------------|-------------|
| | Initial value | Mixed value |
| Aspirate/dispense on Multimek, constant height | — | 39 |
| Aspirate/dispense on Multimek, with tips following liquid level | — | 4 |
| In-house shaker, 100% ink solution | 51 | 51 |
| In-house shaker, 10% ink solution | 48 | 3 |
| Vprep, tips held at bottom during mixing | — | 38 |
| Vprep, top of well dispense only | — | 27 |
| Vprep, tips held at top during mixing | — | 5 |
| Bulk dispense, single addition | 57 | 11 |
| In-house shaker with pin tools | 48 | 3 |

The image in Fig. 12A shows the dispense of ink solution from the fixed-tip Vprep head. In this case the tip inner diameter is much smaller (approximately half the diameter) than that of the disposable tips on the Multi-mek head. However, the lack of motion of the tips correlates to the lack of mixing top to bottom. The image in Fig. 12B shows a more thoroughly mixed state without the vertical folding. The fixed tip jet blends the fluids to an almost visible homogeneity.

The image of Fig. 13 shows the single dispense of the ink solution from Vprep tips set at the top of the fluid. The fluid velocity is such that the DMSO solution is better distributed on the initial dispense, especially compared to multiple aspirate-dispense cycles, at the same velocity, near the bottom of the well (Fig. 12A).

Bulk dispensing

Pressure-driven bulk addition has advantages and disadvantages over syringe systems. The velocity of the manifold better mixes from the start. Figure 14 shows an image of the well after the bulk addition. The ink is well distributed but is not homogeneous, even at the millimeter length scale. Further mixing during this addition step is not possible with this non-contact device. Diffusion is required to mix the fluids at the smaller scales. If immediate and intimate blending of fluids is required in an assay step, this technique may not be adequate.

Comparison of techniques

Table 3 shows the relative amount of mixing for each technique. Analysis of the initial and final state of the well images was done. Where collected, the initial CV value of the unmixed well is approximately 50%. CVs below this value indicate a more homogeneous solution.

These image data show some important issues related to mixing methods. The amount of mixing from a simple dispense is minimal because of the laminar (low Reynolds number) regime. Even though still in the laminar regime, an accelerated jet has the ability to disperse and fold two fluids together and enhance mixing. Multiple aspirate and dispense cycles do not necessarily increase mixing, but when coupled to variable-height folding, mixing can be enhanced. As seen previously, convective mixing is greatly enhanced when shaking is replaced with stirring.

These beneficial techniques have limitations. Assay conditions may limit the use of certain devices, devices may be physically limited in function, and biological and chemical timeframes may conflict with optimal processes, but attention to the efficiency of the method may produce more consistent and reliable assay data.

Conclusions

Mixing in 384-well plates is different than in 96-well plates. The Weber, Reynolds, and Froude numbers do not scale with each other with a change in well dimension. Attention to and revision of the method used to mix can improve solution homogeneity, and therefore data quality. Stirring is more efficient compared to shaking. We have shown two methods to quantitate mixing through image analysis and bottom reading. These methods are precise enough to distinguish mixed from unmixed, and states in between. We have suggested new methods to improve mixing: vary aspirate and dispense heights and shake while pin tools and/or pipette tips are in the wells. Application of these methods may depend on the specific requirements and parameters of the assay. Thorough understanding of the practical aspects of mixing will allow more consistent and repeatable data across many biological and chemical processes.

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