Automating the Dropping Process to Generate Quality Metaphase Spreads in Preparation for Fluorescence In Situ Hybridization (FISH)

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ABSTRACT

Cell 'dropping' refers to the process of applying patient cell samples to a microscope slide in preparation for fluorescence in situ hybridization (FISH). Harvested cells, suspended in a 3:1 (methanol: acetic acid) fix solution, are applied to glass microscope substrate. When applied to the slide, the objective is to have the 'dropped' cells spread in such a way that cellular DNA is presented properly for further analysis using FISH. In the case of Interphase FISH, spreading requirements are not as stringent. In the case of Metaphase FISH, however, condensed chromosomes need to be distinct, the proper size, and separated from neighboring metaphase cells. Historically, the dropping process has been done manually in cytogenetic laboratories and while the definition of a quality metaphase is widely accepted, laboratories often differ greatly with respect to the dropping conditions. The process to date is still inherently manual, and is often viewed as an art, rather than a science. Using the BioJet Plus™ dispensing technology, a quantitative, non contact, liquid dispensing system with drop volumes in the range of 10-4.0 nl, we demonstrated the transition to a consistent automated method, thus eliminating the "art" in the process.

The BioDot AD1500 configured with the BioJet Plus™ technology was used to evaluate the automation of the dropping process and produce consistent, quality, metaphase spreads. Control slides were prepared with the standard manual process (protocol supplied by Signature Genomics) and were used to establish quality standards for the BioJet Plus technology. All slides were evaluated using a Phase Microscope. Experimentally, we have explored the effect of dispense volumes (350 nl to 2 ul), surface temperature (30˚C to 70˚C), surface angle depositions (0˚ to 60˚), surface pre-treatments, local relative humidity (45% to 70% RH), and how they impact a quality metaphase spread. In doing so, an automated protocol was developed that produced metaphase spreads that are sufficient for FISH analysis. We have described key elements of the automated dispensing system that was developed to automate the dropping process. In addition to automating the dropping process, drop volumes have been reduced to the point where multiple cell spots can be applied to a single glass slide allowing for high throughput multiplexing. These additional cell spots can be duplicate spots (the same patient sample) or different patient samples.

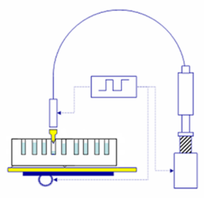
INTRODUCTION

Many cytogenetic laboratories have developed specific protocols to ensure their dropping methods are repeatable; however, there are still many challenges to dropping manually, including operator-to-operator variability, pipette error, and lack of control over environmental conditions, which have made the process very difficult to control. This inherent variability plays a significant role in the quality of metaphase cells on the slide. Metaphase FISH and karyotyping require that the chromosomes from metaphase cells spread adequately so that they can be properly examined by a technician. Since this is a mechanical process, repeating the conditions that affect the cell spreading phenomenon, such as drop volume, slide temperature and local relative humidity, is critical. Some compare cell dropping to breaking an egg: how you crack and drop the egg, i.e. the cell, will determine how the egg yolk and contents, i.e. metaphase, are delivered. As long as the technique is repeatable, you can expect similar looking "eggs" every time.

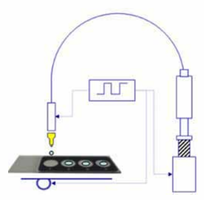
The BioDot BioJet Plus™ dispensing technology has been used to automate the slide dropping process for fluorescent in situ hybridization (FISH). In addition to developing an automated workstation for the application of patient cells to glass microscope slides, the dispense method is capable of jetting droplets as low as 10nl in volume. This reduction in dispense volume now makes it possible to apply more than one cell spot per slide; consequently, lab throughput may be further increased, as multiple patients can be applied to one slide.

Automated Cell Dropping Process Using the BioDot AD1500 with BioJet Plus Technology

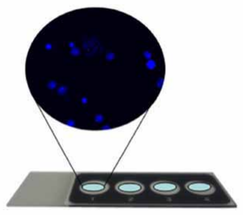
Automatically Aspirate Samples For Source Plate (10 µl)



Automatically Dispense Sample to Slides (500 nl)



Hybridize Slide & Analyze Probes



The optimized cell dropping method dispenses 500nl cell spots. Due to the smaller dispense volume, the drying dynamics are different and therefore a new set of conditions had to be developed; the optimization process required to generate consistent and quality metaphase spreads is detailed below. In addition to generating quality metaphase spreads, each cell spot must contain an appropriate number of cells for labs using FISH in a clinical setting.

The BioDot AD1500: MDx Configuration

The requirements for dropping cells in a clinical environment were determined by Signature Genomic Laboratories:

- 30 quality metaphase cells per spot
- Quality Metaphases: metaphase cells where the chromosomes are spread to the point where each chromosome is distinct (no significant overlap with other chromosomes) and the chromosomes from one metaphase cell cannot spread to adjacent metaphase cells. The metaphase cell membranes should not be visible and individual chromosomes should be elongated and grey to dark grey in color.

DISCUSSION & CONCLUSION

The BioJet Plus™ Technology has been optimized to consistently generate metaphase spreads for the clinical laboratory setting. The miniaturization of the dropping volume from 10,000nl to 500nl has enabled high throughput capabilities in the area of FISH and karyotype analysis. Multiple patients can now be applied to a single slide with an automated dispensing system, which will have a significant impact on throughput by improving technician output.

Automated microscopes have been developed to automatically scan slides for cells and interpret fluorescent probes for diagnosis. These microscopes are slow because they scan the entire surface of the slide to capture all potential cells. Our cell spotting technique can potentially integrate with automated microscopes and 'tell' them where the cells are on a slide. This would significantly increase the speed of automated microscopes, and establish the basis for a fully automated FISH lab.

METHODS & RESULTS

Initial Dropping

Patient cells were harvested on the Hanabii-PIII: Metaphase Harvester at SGL and suspended in 3:1 fix (methanol:acetic acid). Standard cell pellets were resuspended in 1ul of fix. Slides were prepared and dropped via the protocol supplied by Signature Genomics using the AD1500 dispensing system with BioJet Plus™ technology. Slides were heated to 70˚C in water, dried, and then applied to the AD1500. The instrument automatically aspirated 20ul of the patient sample from a source well, then dispensed 24 unique spots on a single slide (100nl per spot). Relative humidity was 45% and the room temperature was measured at 23˚C. Cells were dispensed onto slides that were held at 45˚C. To approximate the 'fix flood' technique used at Signature Genomics, the instrument was programmed to apply a drop of 3:1 fix solution to the slide just before the patient cell spot was applied. Spotted cells were imaged using an Olympus Phase microscope, Phase 2 filter, 10x and 40x objectives.

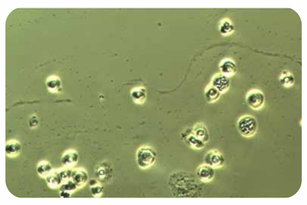


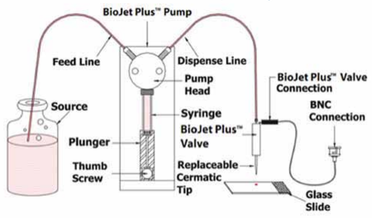
Image of cell spots generated by the BioDot AD1500 (Olympus BX40 Phase Microscope, Phase 2, 10X objective). Drop volume was 100nl.

All observed cells were very "shiny," indicating poor spreading. The cell membranes of the metaphase cells were clearly intact. In addition, the chromosomes from metaphase cells were highly reflective and could not be individually identified due to a high level of overlapping chromosomes. Furthermore, the number of metaphase cells per spot was not sufficient to meet the 30 cell requirement. Overall, the observed cells appeared to have dried too quickly and were not in a sufficient concentration.

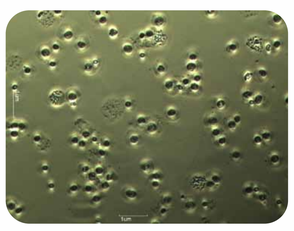
Drop Volume

The BioJet Plus™ Dispensing technology is a non-contact dispensing technology used to precisely print biological solutions onto surfaces. The BioJet Plus™ dispenser couples a high resolution syringe pump and a microsolenoid valve to dispense 10nl to 4000nl droplets. This high speed, high precision technology has been used to dispense cells, proteins, and DNA onto various substrates in order to build low volume assays. In addition to providing an automated means for dispensing patient cells, this dispenser was selected because it is capable of dispensing multiple spots on a single side.

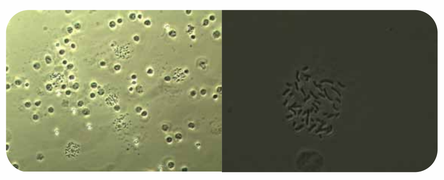
BioJet Plus™ Dispensing



Drop volumes were tested between 100nl and 1000nl. 1000nl was chosen as a maximum volume in order to maintain the ability to multiplex. Larger volumes would not allow us to place more than one spot onto a glass slide. Metaphase spreads were observed from 100nl, 250nl, 500nl, and 1000nl cell spots.



100nl dispense volume, observed using an Olympus BX40 Phase Microscope, Phase 2, 10x objective

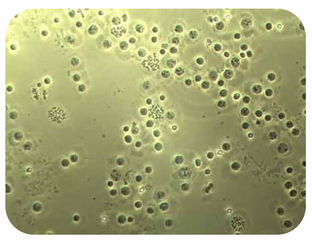


500nl dispense volume, observed using an Olympus BX40 Phase Microscope, Phase 2, 40x and 10x objectives

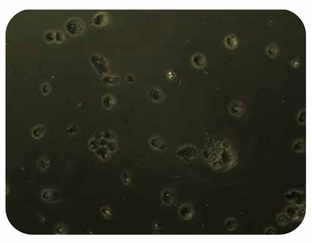
Only 500nl and 1000nl spots produced adequate spreads. Cell spots below 500nl continued to show high levels of reflection, significant amounts of cytoplasm, and unacceptable chromosome overlap.

Slide Temperature

An aluminum slide holder was designed so that heated water could circulate through the holder and therefore control the temperature of the slide surface. Slides were again dropped with the AD1500 workstation. The effects of slide temperature were observed as slide temperature was adjusted from ambient temperature, 24˚C, to 45˚C. Slide angle was 0˚, drop volume was held constant at 1ul, and relative humidity within the instrument was programmed and held constant at 65%. Tests were performed in increments of 5˚C.



Slide temperature (30˚C), angle (0˚), dispense volume (500nl), RH (65%),:1 Methanol:Acetic Acid was used to pretreat the slide surface. Observed using an Olympus BX40 Phase Microscope, Phase 2, 10x objective



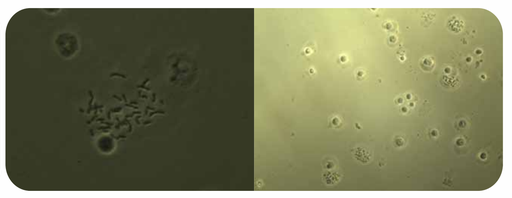
Slide temperature (40˚C), angle (0˚), dispense volume (500nl), RH (65%), 3:1 Methanol:Acetic Acid was used to pretreat the slide surface. Observedusing an Olympus BX40 Phase Microscope, Phase 2, 10x objective

Slides heated to 30˚C produced the best metaphase spreads.

Slide Surface Pretreatements

Signature Genomic uses a 'fix flood' process, meant to aid chromosome spreading on the slide; prior to dropping patient samples on the slide. In order to investigate the effect of 'fix flooding' for our miniaturized FISH technique, slides that were not treated with fix prior to spotting were compared with slides that had fix applied to the slide just before spotting patient cell spots. Superfrost glass micro slides (VWR, precleaned) were used in these experiments. Slide conditions held constant included: dispense volume (500nl), RH (65%), slide temperature (30˚C), and slide angle (45˚). Patient samples from slides that were not treated with a "fix flood," slides that were treated with a 3:1 methanol to acetic acid "fix flood," and slides treated with a 1:1 methanol to acetic acid "fix flood" were examined. 1000nl of "fix flood" treatment solution was applied to the slide in the same location as the eventual patient spot. The "dwell time" of the treatment solution on the surface before applying the patient spot was approximately 1 second.

No pre-treatment spots were applied, 500nl dispense volume, observed using an Olympus BX40 Phase Microscope, Phase 2, 10x objective



1:1 methanol to acetic acid applied to the slide prior to dispensing the sample. 1.0ul dispense volume, observed using an Olympus BX40 Phase Microscope, Phase 2, 40x and 10x objectives

Slide Angle

The protocol at Signature Genomic calls for slides to be angled at 60˚ angle during the dropping process. Patient slides were produced using a 60˚ slide angle and a 0˚ slide angle in order to determine the need for such an angle with the automated system. The 60˚ was created by placing one edge of the slide on the slide container until the 60˚ angle was achieved. Slide conditions held constant included: dispense volume (500nl), RH (65%), slide temperature (30˚C), angle (60˚), 1:1 methanol:acetic acid was used to pretreat the slide surface.

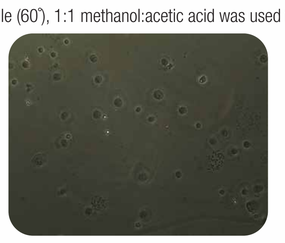


Image of cell spots generated by the BioDot AD1500 (Olympus BX40 Phase Microscope, Phase 2, 10X objective). This slide was dispensed with no angle (0˚)

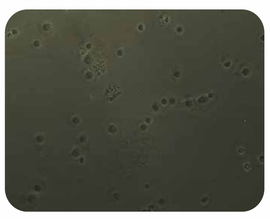


Image of cell spots generated by the BioDot AD1500 (Olympus BX40 Phase Microscope, Phase 2, 10X objective). This slide was dispensed a 60˚ angle.

No significant difference was observed.

Relative Humidity

The localized humidity near the dropping area is considered to be a critical aspect of the dropping process. The Signature Genomic dropping protocol calls for 45% RH while dropping patient cells. Slides were dropped between 45% RH and 70% RH. Slide conditions held constant included: dispense volume (500nl), RH (65%), slide temperature (30˚C), 1:1 methanol:acetic acid was used to pretreat the slide surface, and the slide angle was 0˚. Humidity was generated and controlled via the automated humidity control system that is integrated into the AD1500 dispensing system. Due to the speed of evaporation from smaller drop volumes, results were expected to improve as humidity was increased.

Image of cell spots generated by the BioDot AD1500 (Olympus BX40 Phase Microscope, Phase 2, 10X objective). This slide surface was increased to 45˚C.

Image of cell spots generated by the BioDot AD1500 (Olympus BX40 Phase Microscope, Phase 2, 10X objective). This slide surface was increased to 30˚C.

Fully Optimized Conditions

The results of our tests indicated that the lower dispense volume caused the cells to dry too fast. A summary of our results is below:

- Dispense Volume: A larger drop volume resulted in improved metaphase spreads.
- Slide Surface Temperature: Cooler slide temperatures improved metaphase spreads.
- Relative Humidity: Increased %RH improved metaphase spreads.
- Slide Surface Pre-treatement: Increased the water content (1:1 methanol to acetic acid has a greater water content than 3:1 menthanol to acetic acid which has a greater water content than no pretreatment) on the slide prior to dispensing the patient suspension improved metaphase spreads.
- Slide Angle: Slide angle did not appear to have an impact on metaphase spreads, as this parameter does not likely play a significant role in overall slide drying times.

Although, during the optimization process, cells with the appearance of drying too quickly were most often observed, it is worth mentioning that in experiments where conditions progressed past optimal ranges, cells with the appearance of drying too slowly were also observed. Because each of the above conditions are interrelated, despite the findings during the optimization process, applying those conditions together did not necessarily produce optimal results.

The correct starting concentration of cells will result in the proper number of quality metaphases per spot. Signature Genomics typically resuspends a harvested sample pellet in 1ml of fix (3:1 methanol:acetic acid). Through a series of experiments where the starting concentration was varied and correlated to metaphase quality, it was found that resuspending a typical sample pellet in 250ul of fix (3:1 methanol to acetic acid) produced the optimal number of metaphase spreads per spot, while maintaining metaphase quality.

After a series of experiments performed to further optimize the process, the following conditions produce the desired result: a repeatable method of generating quality metaphase spreads.

- Cell Spot Volume: 500nl
- Cleaned slides: using the SGL FISH slide cleaning protocol.
- Slide Surface Temperature: 31*C
- Relative Humidity: 65%
- 1:1 methanol:acetic acid pretreatment volume: 3000nl
- Slide Ange: 0*
- Starting Concentration of Cells: Typical Pellet Suspended in 250ul of Fix (3:1 methanol to acetic acid)

The above protocol and set of conditions were used on multiple slides over multiple days. The dropped slides were then taken through the complete FISH protocol. Hybridization solutions were automatically applied using the BioDot AD1500. The results are below:

Image 1: Metaphase cell labeled with X Centromere (green) and Y Centromere (red) probes. Cells counterstained with DAPI plus Vectashield. Image taken with the Zeiss Axio Imager.M1 using the 100x Oil objective.

Image 2: Metaphase cell labeled with X Centromere (green) and Y Centromere (red) probes. Cells counterstained with DAPI plus Vectashield. Image taken with the Zeiss Axio Imager.M1 using the 100x Oil objective.

Image 3: Metaphase cell labeled with X Centromere (green) and Y Centromere (red) probes. Cells counterstained with DAPI plus Vectashield. Image taken with the Zeiss Axio Imager.M1 using the 100x Oil objective.

Image 4: Male and female metaphase and interphase cells labeled with X Centromere (green) and Y Centromere (red) probes. Cells counterstained with DAPI plus Vectashield. Image taken with the Zeiss Axio Imager.M1 using the 20x objective.

Image 5: Male and female metaphase and interphase cells labeled with X Centromere (green) and Y Centromere (red) probes. Cells counterstained with DAPI plus Vectashield. Image taken with the Zeiss Axio Imager.M1 using the 20x objective.

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