Improved Karyotyping of Low Volume Blood Cell Cultures

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Abstract:

Karyotyping is used for chromosome analysis. Common practice involves syringes with needles and the removal of blood from the brachial vein in the arm. This invasive method requires about one milliliter of blood for one karyotype. This research expands on previous work, and attempts to improve the quality of the chromosome spreads obtained from low blood volumes, approximately twenty microliters, and to obtain images that allow for karyotyping. The results produced are comparable to those from the one milliliter of blood. For both healthcare and for research, this method has practical applications. The procedure is less invasive, using only a finger-prick to obtain samples. The imaging techniques have been improved upon and banding techniques used for staining. The spreads imaged have banding patterns, making them candidates for karyotyping once the patterns are clearer.

Introduction:

A karyotype is the picture arrangement of the chromosomes of a single cell, but more recently the definition has expanded, and now includes written descriptions of the images. The chromosomes are arranged on the karyotype in a specific order. They are grouped by size, morphology and centromere location (Richardson, 1997).

The convention, in looking at chromosomes, is to have the short p-arm at the top. Metacentric chromosomes have the centromere near the middle, acrocentric chromosomes have the centromere near to one end, giving very short p-arms, and sub-metacentric chromosomes have the centromere at a position intermediate to these two extremes. The International System for Human Cytogenetic Nomenclature (ISCN) separated the chromosomes into seven groups. The twenty two autosomal pairs and the sex chromosomes are each placed into one of the seven groups as outlined below:

In group A, the chromosome pairs are one to three, which are large, metacentric, and can be distinguished from each other based on their size and their centromere position. Group B contains pairs four and five, which are large, sub-metacentric, and are not easily distinguished from each other by simple staining procedures. Group C contains pairs six through twelve and the X chromosome. These are metacentric chromosomes that are medium sized and difficult to distinguish from each other by simple staining procedures. Group D contains pairs thirteen to fifteen, which are medium, acrocentric chromosomes. Group E is made up of pairs sixteen to eighteen, which are short chromosomes, but can be distinguished from each other based on their centromere position: pair sixteen is metacentric and pairs seventeen and eighteen are sub-metacentric. Group F is made up of pairs nineteen and twenty, short, metacentric chromosomes. Group G contains the remaining two pairs of autosomes, twenty-one and twenty-two, as well as the Y chromosome. The pairs are short and acrocentric, with satellites. The Y chromosome is the same size and shape as the group G chromosomes, but is distinguished because it has no satellites. Chromosome pair twenty-one is actually shorter than pair twenty-two (Wolstenholme, 1992).

Further chromosome identification is accomplished by banding. A band is the part of the chromosome that is distinguished from adjacent segments through staining techniques such as Giemsa banding and Quinicrine banding, making them appear darker or lighter. The bands are unique to each chromosome pair. For convenience, the chromosome is divided into regions that are marked by landmarks, which are fixed points, such as the telomere and centromere, and some prominent bands. The region is numbered sequentially, moving outwards from the centromere. This naming helps to identify locations on the chromosomes and also helps to identify the chromosome (Wolstenholme, 1992).

The process of acquiring a karyotype begins with cell culture. Culturing allows cells to proliferate *in vitro*, or outside of the body. This can be more desirable because cultured cells can be manipulated more easily than cells that proliferate *in vivo*, or inside the body (Nichols *et al.*, 1979). These cells are able to proliferate *in vitro* because they are grown in medium, which consists mainly of an isotonic solution of water and salts (Mather *et al.*, 1998).

The cells being cultured are white blood cells, or lymphocytes. Mature red blood cells do not have chromosomes because they lack nuclei, and are thus of no importance to the focus of the experiment. The chromosomes contained in the nucleus of the lymphocytes make them excellent candidates for use in this experiment, and the cells are easily manipulated during culture and harvesting to give clear chromosome spreads upon dropping onto a microscope slide. Lysing the red blood cells could potentially improve the quality of the spreads because there would be less debris, which is due to the red blood cells. Erythrocyte lysis solution (ELS), a ProCell reagent, is gentle in comparison to similar products, and should not have an effect on white blood cells (Rainbow Scientific, 2006).

The medium that was used is PB-Max Karyotyping medium (Gibco #0552). It contains fetal bovine serum, L-glutamine, and phytohemagglutinin (PHA). This medium is specifically designed for use with peripheral blood sample cultures. The fetal bovine serum is blood without cells or clotting factors, and serves as a source of minerals, lipids, and hormones. The serum promotes proper growth circumstances for the cultured cells because of the presence of growth factors (Freshney, 2000). PHA, a mitogen, stimulates mitosis in the lymphocytes. PHA is one of the most used mitogens in blood cell cultures because of the positive results that have been observed with it (Gosden *et al.*, 1992). L-glutamine, an amino acid, is also important to the culture medium. Glutamine is required by most cells and is used in

cultures as an energy and carbon source (Butler and Christie, 1994). The L-glutamine is used by rapidly dividing cells, such as lymphocytes (Greenwell, 1999).

The culture itself must be prepared in a sterile environment, and every effort to avoid contamination must be made. Ethanol is often used to keep the working environment clean when working in the laminar flow hood. Antibiotics, such as penicillin or streptomycin, work to help fend off bacteria that could threaten the culture's survival (Gosden *et al.*, 1992), and are sometimes included in the medium. PB-Max contains the antibiotic gentomycin. Other precautions include having hair tied back, wearing a mask if ill, not placing objects in front of one another, and not crossing an arm or an object over an open bottle. Sterility is not only imperative to the experiment's success, it is also important for safety when handling blood. Gloves should be worn at all times when handling the blood cultures (Freshney, 2000).

Cellular growth has very specific requirements for success. Incubators are used to mimic some physiological aspects of the human body. Each medium has specific requirements, and the CO_2 setting should be matched to the medium being used. This setting provides the concentration of CO_2 necessary to work with the bicarbonate buffering system that regulates the pH of the culture. The carbon dioxide, as well as the bicarbonate buffering system, provides a healthy environment for the cells to grow in. The pH of the body, *in vivo*, is usually between 7.0 and 7.4. If the carbon dioxide concentration does not agree with the specifications of the medium being used for the culture, the pH could fall out of the healthy range for the cells, causing cell death and loss of the culture (Mather *et al.*, 1998).

Chromosome resolution additive (CRA) prevents chromosome contraction, making the chromosomes more elongated in appearance by loosening the chemical bonds in the supercoiled structure. This solution can be added pre or post colcemid (Rainbow Scientific, 2006). Past research has shown that the best time to observe chromosomes is during metaphase, because the chromosomes are maximally

condensed. This stage can be achieved in culture by using a chemical to stop the mitotic division of the cells at this stage. Colcemid is used to stop mitosis, and is added to the culture prior to harvesting. Colcemid is a form of colchicine, and is an experimental drug which binds tubulin, the protein that controls microtubule assembly. The mitotic spindle is made of tubulin, and its formation is inhibited in the presence of colcemid, which in turn blocks mitosis of the cell (Cooper, 1997). Metaphase arresting solution is a mixture of colchicine and vinblastine sulfate in PBS. Both of these inhibit mitotic spindle formation and increase the percentage of cells in metaphase (Rainbow Scientific, 2006). This is another option, to be used in place of colcemid, to halt mitosis in the culture prior to harvesting.

Prior to being harvested, the cultured cells are treated with a pre-hypotonic solution. This solution, a ProCell Reagent, makes the hypotonic treatment of the cells more effective by helping to prevent clumping of the chromosomes. The clumping of the metaphase chromosomes can inhibit spreading. This treatment thus optimizes the hypotonic treatment, which causes the cells to swell, increasing the cell volume and allowing the chromosomes to spread out, giving better spread chromosomes upon analysis and fewer cross-overs (Rainbow Scientific, 2006). The optimal hypotonic solution from ProCell Reagents was used in conjunction with their pre-hypotonic solution.

Following the hypotonic treatment, the cells are fixed. The fixative consists of one part glacial acetic acid, three parts anhydrous methanol, and cytoclear, a ProCell Reagents product. The cytoclear is a mixture of protein detergents that dissolve and remove most of the cytoplasm during the fixation stage of harvesting. It dissolves the lipid and glycoprotein structure that predominates in the cytoplasm. This helps the chromosomes spread better when dropped. Adding this to the fixative, the solution has to stand for 10 minutes before being centrifuged, the supernatant

discarded, and the cells resuspended in the fixative (Rainbow Scientific, 2006). This is the harvesting step.

After harvesting is complete, the cells must be dropped onto slides. It was found that two feet was the best height to drop the slides from, and they resulted in the best spreads when dropped onto a slide that was placed flat, on ice (Werst, 2005). Dropping the cells gives the chromosomes the capacity to spread further apart. The further apart the chromosomes are, the easier it becomes to stain and image them afterwards. An ideal spread is one that contains no overlaps of chromosomes and has them well spread, which can ultimately result in a karyotype.

Among the many different staining methods, Giemsa staining and Giemsa banding (G-banding) were the main focus of this experiment. Staining the chromosomes with a Giemsa stain, not considering banding, does not require an enzyme digest, and stains the entire chromosome a deep blue color. The Azure B and the Eosin in the Giemsa bind to the DNA in the chromosomes, staining them (Benn and Perle, 1992). G-banding is more complex in nature, and takes advantage of structural elements of the underlying DNA in the chromosomes.

G-banding has become one of the more widely used methods for staining chromosomes. The slides are treated with a protease, usually trypsin. The trypsin digests proteins that are associated with the chromosomes, allowing the stain to produce the banding patterns seen. Trypsin has certain difficulties, because each batch of trypsin is slightly different, requiring experimentation with every batch to produce satisfactory banding (Saitoh, 1994). The dark bands seen in the G-banding are found in regions that are A+T rich. These regions replicate late in the S phase of the cell cycle. It is also thought that these regions contain few active genes and have different protein composition from the lighter bands observed (Benn and Perle, 1992).

Following the staining, imaging of the chromosome spreads must be done before karyotyping can occur. Light microscopy is used for imaging, and the images are taken under oil immersion. When examining the spreads, they are diploid and each chromosome consists of two chromatids due to the arresting of the mitotic process at metaphase. A chromatid is one of the usually paired strands of a duplicated chromosome. The chromatids are joined by a single centromere (Merriam-Webster, 2006). The spread chromosomes can then be arranged into a karyotype (Freshney, 2000).

The camera used was the Optronics Magnafire. This camera, attached to the light microscope, also utilizes its own software to process and view the images, both in real time and after capturing them. The software is capable of many different processes for the images. The white balance is important in correctly determining, for each individual slide, the ratios of green, blue, and red in the captured images, and ensures that the images on the screen show white as white (Optronics). To accomplish this, there is no slide placed in the field and the auto white balance is used. The camera software has many of the same options available in Adobe Photoshop, and either software could be used for image processing. However, in this case Adobe was used due to the ease of manipulation of the image in that software. Auto-leveling corrects the brightness of the image. The brightness and contrast can also be changed manually, if desired, and the images cropped to contain only the desired spread. Sharpening the edges, if needed, can enhance the chromosomes' edges, and sometimes sharpen the bands that are visible in the image.

Karyotypes have many roles in cytogenetic research and in the diagnosis of chromosomal abnormalities. Normally, processes such as amniocentesis and chorionic villus sampling (CVS) are used as prenatal tests of fetal chromosomes. Amniocentesis is an invasive procedure where a needle is passed through the woman's abdomen into the amniotic cavity within the uterus. Fluid is drawn from

there to test the cells for chromosome abnormalities (Prenatal Diagnosis, 2006). CVS involves removing a small sample of the chorionic villi for genetic analysis, and is usually done for women who are at risk of carrying a child with a genetic defect or chromosomal abnormality. Some of the diseases that can be identified using these methods are Kleinfelter's syndrome, Down syndrome, and Tay-Sachs disease. Consent forms are required for these tests due to the personal nature of the information gained (an example of a consent form is included in Appendix B).

Materials and Methods:

Caitlin Halbert (2004) and Lauressa Werst (2005) worked previously on this experiment, and were able to culture low blood volumes and optimize a culturing and harvesting protocol. The following experiments were conducted to improve on their work and optimize the chromosome spreads obtained for imaging, with the intent of creating a karyotype from a satisfactory image of the chromosome spreads.

The materials required for the blood cell culture and harvesting are: PB-Max Karyotyping Medium (GIBCO 0552), Karyomax colcemid solution (GIBCO 0338), pre-hypotonic solution (Rainbow Scientific #GGS-JL-007), optimal hypotonic solution (Rainbow Scientific #GGS-JL 005/a), CRA (Rainbow Scientific #GGS-JL-003/a), fixative (3 parts methanol, 1 part glacial acetic acid), and cytoclear (Rainbow Scientific #GGS-JL-004). Staining requires the following materials: Gurr buffer (BDH #33199), Giemsa stain (GIBCO 068), sterile water, Hank's Balanced Salt Solution (HBSS) (Sigma H6648), fetal bovine serum (FBS) (GIBCO) and 2.5% trypsin (GIBCO 0409).

The medium used to culture the cells was PB-Max, which was purchased already prepared and only required thawing for utilization. All work using the medium was done in the laminar flow hood to maintain a sterile environment. One hundred sixty microliters of the medium was placed into one well of a sterile ELISA plate and then twenty microliters of blood was added to the well. The blood was

collected using spring-loaded pens called lancet devices (B-D Corporation), alcohol towelettes, and a micropipette. Fingertips were cleaned using the alcohol towelettes prior to lancing. They were then pricked with a sterile disposable tip within the sterile lancet device. The tips were properly disposed of in the sharps waste container. The blood was collected using a micropipette and sterile tips, ten microliters at a time. The ELISA plate was then incubated for four days in a CO_2 incubator at 37°C and 10% CO_2.

Following the four days of incubation, two microliters of working CRA solution was added to each well. The working solution of CRA is made by adding one microliter of CRA to ninety nine microliters of sterile water. The CRA was added 2.5 to 3 hours prior to harvesting. Thirty minutes after adding the CRA, eighteen microliters of colcemid solution was added to each well, bringing the total volume in each well to two hundred microliters.

After incubating for another 2 to 2.5 hours, the culture was transferred to a microfuge tube and spun at 2,000 rpm for five minutes. After centrifugation, the supernatant was removed and discarded. Two hundred microliters of Karomax and twenty microliters of pre-warmed pre-hypotonic solution were added to the tube and the pellet was gently mixed by flicking the closed tube. The tubes were then centrifuged for ten minutes at 1,000 rpm.

Following centrifugation, the supernatant was removed and discarded. Two hundred fifty microliters of pre-warmed optimal hypotonic solution was added to each tube drop by drop, folding the pellet into the solution. Folding is the gentle, repetitive overturning of the mixture without beating or stirring (Merriam-Webster, 2006), and allows for the incorporation of the hypotonic solution into the cells. Any liquid remaining in the pipette tip was expelled into the solution and the solution was then incubated in the CO_2 incubator at 37°C for twenty minutes.

During this incubation period, the fixative was prepared by mixing one milliliter of acetic acid and three milliliters of methanol in a conical tube. Twenty microliters of cytoclear was added to the conical tube. This solution was then vortexed to mix. It may be necessary to make more fixative: four milliliters serves for eight wells.

After the incubation, the solution was centrifuged for five minutes at 2,000 rpm. The supernatant was removed and discarded. Two hundred fifty microliters of the fixative was added drop by drop, folding the pellet into solution as before. Any remaining solution in the pipette tip was expelled into the solution. The pellet turns a dark brown color at this point. The solution was left to stand for ten minutes. The solution was then centrifuged for five minutes at 2,000 rpm. The pellet is clear to light brown and difficult to see, so it is important that proper procedures are followed while centrifuging. The hinge of the microfuge tube should point up, giving a reference point for the location of the pellet. The supernatant was removed and discarded. The pellet was then resuspended in two hundred fifty microliters of fixative using the folding method to mix.

Prior to dropping, the slides were soaked in 95% ethanol for fifteen minutes and allowed to dry. After they were dry, the slides were placed as flat as possible on ice. The suspended cells were then dropped using a Pasteur pipette from a height of approximately two feet onto the slides. The slides were allowed to dry for at least twenty four hours prior to any staining.

For Giemsa staining without banding, the slides were stained with 100% Giemsa stain. The slides were completely dry before this staining was done. The slides were dipped in Gurr buffer for thirty seconds and then allowed to dry. The slides were then covered with one milliliter of Giemsa stain and sat for ten minutes. The stain was rinsed off of the slide with deionized water and the slides were allowed

to dry. The dry slides were made permanent using Cytoseal 60 (VWR) and a coverslip.

The G-banding required aging of the slides. The slides were placed in a dry oven, set to between 55°C and 60°C, and left overnight. The slides were removed from the oven and brought to room temperature just before banding. The trypsin working solution is 1.5 milliliters of 2.5% trypsin solution in fifty milliliters of Hank's balanced salt solution (HBSS), the fetal bovine serum solution is one milliliter of fetal bovine serum in fifty milliliters of HBSS, and the Giemsa stain working solution is one milliliter of Giemsa stain in fifty milliliters of Gurr buffer, pH 6.8. This procedure also required a buffer rinse of Gurr buffer, pH 6.8, and a water rinse of sterile water. The slides were dipped in the trypsin for eight seconds using a back and forth motion to expose the slide to the solution. It was important to keep track of which side contained the dropped cells. The slide was removed from the trypsin and quickly dipped in the fetal bovine serum solution, using the same motion as before, for five seconds. This deactivated the trypsin. The slide was pre-rinsed in the buffer rinse by dipping for five seconds as before. The slide was placed in the Giemsa stain working solution for ten minutes. After staining, the slide was dipped in the water rinse for five seconds using the same technique as before. The slide was left to air dry. Once dry, a coverslip was added using Cytoseal 60 (VWR). The coverslip and mounting medium were left to dry overnight before imaging the slides. Prior to imaging, auto-white balancing was done using the software that accompanies the camera. The slides were viewed under a light microscope and photographed using an Optronics Magnafire camera.

The slides observed were evaluated for quality spreads and the images captured were then processed for optimization in Adobe Photoshop. The image was auto-leveled, sharpened, cropped to the desired size, which is specific to each image, and the contrast and brightness were auto-adjusted.

With every alteration to the standing protocol, information was gained that aided further experimentation, and helped the project progress successfully. There were several small experiments done to evaluate the different reagents that were considered for addition to the protocol. The different reagents evaluated were erythrocyte lysis solution (ELS), chromosome resolution additive (CRA), cytoclear, metaphase arresting solution, the pre-hypotonic solution, and the optimal hypotonic solution.

The ELS was added to two wells on the day of the start of the culture, and to two other wells on day three of the culture. The harvesting was then done on day four. The procedure for ELS was to pipette twenty microliters of blood into a microcentrifuge tube and add one hundred sixty microliters of PB-Max medium. This solution was spun at 1,000 rpm for five minutes. The supernatant was removed and discarded, and the pellet was resuspended in fifty microliters of ELS. This solution was spun at 1,500 rpm for five minutes. The supernatant was removed and discarded, and one hundred microliters of PB-Max medium was added to rinse the pellet. This solution was centrifuged at 1,500 rpm for five minutes. The supernatant was removed and discarded and the pellet was resuspended in one hundred eighty microliters of PB-Max medium. This solution was then moved to the assigned well on the ELISA plate. It was found that the ELS was too strong for the volume of blood being used, and lysed all cells in the culture, not just the erythrocytes, resulting in slides with no chromosome spreads upon staining. ELS is typically used to clarify large blood samples. Since it did not work on this scale its use was discontinued.

Metaphase arresting solution was also tested in comparison to the colcemid. Out of the eight wells in the plate that contained cultures, four had eighteen microliters of metaphase arresting solution added to them, and the other four had eighteen microliters of colcemid added. At first, the metaphase arresting solution gave better chromosome spreads than the colcemid. However, in subsequent

experiments it was no longer as effective as the colcemid. It had reached the end of its shelf life, so was no longer used. Since the colcemid gave satisfactory results, it remained the mitotic inhibitor in the protocol.

The CRA was added to half of the wells, two microliters per well, thirty minutes before the colcemid was added. This gave many quality spreads, with little crossover. The overall observation of the slides without CRA was a mix of quality spreads and non-quality spreads, which contained many crossovers, clumping, or very condensed chromosomes, making identification for karyotyping difficult. Because the CRA slides gave more quality spreads on each slide, use of the CRA was kept in the standing protocol.

The cytoclear added to the methanol:acetic acid fixative gave better spreads than the fixative alone, as the cytoplasm no longer inhibited spreading. Some of the spreads were so well spread that it was difficult to distinguish which chromosomes belonged to which cells in the area. A karyotype would still be able to be constructed, however.

In evaluating the optimal times for the trypsin digest and the Giemsa stain for the G-banding protocol, four slides were used. For the trypsin digest time, the stain time was held constant at ten minutes for all four slides and the digest time varied. The digest times were five, eight, ten, and fifteen seconds. For the Giemsa stain, the optimal time for the trypsin digest, found to be eight seconds, was used for all four slides, and the stain times varied. The stain times were five, eight, ten, and fifteen minutes. The optimal stain time for the Giemsa stain, for G-banding, was found to be ten minutes.

A laboratory protocol has been included in Appendix C.

Results:

The modifications to the current procedure (Werst, 2005) occurred in many steps. With every alteration, information was obtained that helped the experiment

progress toward better spreads and clearer images. The experiments involved additions to the current procedure or replacing reagents in the current procedure, with the ultimate goal of chromosome spreads that could be used to create a karyotype. Erythrocyte lysis solution (ELS), metaphase arresting solution (MAS), chromosome resolution additive (CRA), cytoclear, pre-hypotonic solution, and optimal hypotonic solution were all tested in the procedure. All of these reagents were provided by Rainbow Scientifics. The spreads were observed under a microscope to determine the effectiveness of each reagent.

The ELS was added to the blood samples before harvesting either on the day of collection or the day before harvesting. It was found that ELS was too strong for the scale of this work. All of the cells, lymphocytes and erythrocytes, were lysed, resulting in slides with no spreads and very few, if any, intact cells. The ELS was abandoned in future experiments as it produced no spreads.

Colcemid is the mitogen that has been used in the standing procedure. MAS contains colchicine, which colcemid is a form of, and vinblastine sulfate in PBS, and was used to replace colcemid. In the experiment, half of the wells containing blood samples and medium received colcemid, as per the standing procedure, and half received MAS. The MAS resulted in more spreads than colcemid, but after subsequent use replacing the colcemid in the standing protocol it was discovered that it was at the end of its shelf life and was no longer effective. As the colcemid still produced quality spreads, it was reincorporated into the protocol.

In another experiment, CRA was added to half of the wells containing blood samples and medium before the harvesting began. The other half received no CRA. It was found that CRA gave a higher number of quality spreads per slide when compared to slides that were not treated with CRA, and that the spreads that were obtained on the CRA treated slides were less condensed than the chromosome spreads on the untreated slides. Less condensed chromosomes are more desirable,

especially when the staining procedure involves banding, because the bands are more clearly visible on uncondensed chromosomes. As these bands can be used in identification of the chromosome as landmarks, this aids in the creation of karyotypes. CRA was incorporated into the protocol, added to the wells thirty minutes before the colcemid on the day of harvesting.

The pre-hypotonic solution and the optimal hypotonic combination resulted in more spreads than slides where only hypotonic solution was used. The spreads obtained from the use of both the pre-hypotonic and the optimal hypotonic solutions were compared to spreads obtained by Lauressa Werst. Those spreads were treated with pre-warmed, sterile hypotonic solution (0.075 KCl) (Werst, 2005). The spreads contained fewer crossovers when the pre-hypotonic and the optimal hypotonic solutions were used. Fewer crossovers give spreads that are better for karyotyping. Both solutions were incorporated into the standing procedure.

In addition to being less condensed (the CRA does this to the chromosomes), the chromosomes needed to be well spread. Although the pre-hypotonic and optimal hypotonic solutions aided in better spreads with fewer crossovers, another reagent was added to the procedure in an attempt to obtain better spread chromosomes. Cytoclear was added to the fixative (3 methanol:1 acetic acid) and compared to using the fixative alone. Addition of cytoclear was found to give better spreads because there was less inhibition of the chromosomes by the cytoplasm. Because the chromosomes were better spread and contained few cross-overs, the cytoclear added to the fixative was incorporated as a permanent part of the procedure.

The trypsin digest time was determined by varying the trypsin digest time of four slides and keeping the stain time constant. The digest times were five, eight, ten, and fifteen seconds. The protocol for the G-banding (NIH, 2005) indicated that the optimal digest time was between eight and ten seconds, depending on the batch of trypsin. The stain time was held at ten minutes. As is shown in Figure one parts

a-d, eight seconds was determined to be the optimal digest time for this batch of trypsin. Based on this information, an optimal stain time was determined by holding the digest time at 8 seconds and varying the stain time. The stain times used were five, eight, ten, and fifteen minutes. The images obtained did not clearly show what stain time was optimal, but under the microscope it was clear that ten minutes was the optimal stain time. Eight seconds of digesting and ten minutes of staining were set as the procedure for the G-banding, but the trypsin digest time and possibly the staining time should be re-evaluated for each new batch of trypsin (see Figure 1 in Appendix A).

The processing of each image was kept standard, so that every image was processed the same way, with the exception of the size they were cropped to, as each spread varied in its size. The image processing was done because the spreads viewed under a microscope were not translating into images that clearly showed the banding of the chromosomes. The processing involved auto-leveling the images, sometimes adjusting the brightness and contrast, sharpening the edges of the image, which enhanced the differences in the chromosomes if there were crossovers in the spreads. In addition, some of the images were cropped so that only the chromosome spread was contained in the image. Examples of quality, banded chromosome spreads that are possible candidates for karyotypes can be seen in Figure two (Appendix A).

Discussion:

The ultimate goal of the project was to optimize the staining and imaging procedures for chromosome spreads. By modifying the current procedure for culturing and harvesting blood cells with the addition of the CRA, cytoclear, pre-hypotonic and optimal hypotonic solutions, the quality of the chromosome spreads was optimized. Once the chromosomes were well spread and contained very few crossovers, the staining of the chromosomes was most important. If clear bands can

be obtained, the chromosomes can be easily distinguished from each other, making the spreads candidates for karyotyping.

In the course of running the different experiments, different donors met with different levels of success. Although results were eventually obtained for each experiment, some of them required several trials before there were spreads that could be assessed. It was found early in the course of this project that frosted slides were best, and they should be labeled with pencil only. The different components of the marker that was being used on unfrosted slides had a tendency to run onto the slides once the cells were dropped onto the slide, as the cell solution covered the entire slide. These components were strong enough to compromise the spreads, so that few, if any, were seen on the slide. Even still, the pencil can sometimes find its way onto the slide, as the solution covers the entire slide, but it does not compromise the spreads as the marker did. Certain donors did not culture or harvest well, and few spreads were observed from their cells, and those that were found were not well spread. There is no explanation for this in the terms of the procedure itself, as other donors produced quality spreads in high numbers. These poor donors were not asked to donate again.

The reagents themselves presented certain problems. It was thought that using the erythrocyte lysis solution (ELS) would solve the problem of debris from broken red blood cells seen on the slides. The ELS was too strong for the scale of the experiment. All of the cells in the culture were lysed, instead of just the red blood cells. It was found later in the course of the experiments that the cytoclear helped clear most of the red blood cells from the culture, acting on them as it acts on the cytoplasm of the lymphocytes. The cytoclear is a protein detergent, and dissolves the proteins that make up the cytoplasm. Clearing the red blood cells out of the culture greatly reduces the debris on the slides, giving the chromosomes more space to spread upon dropping.

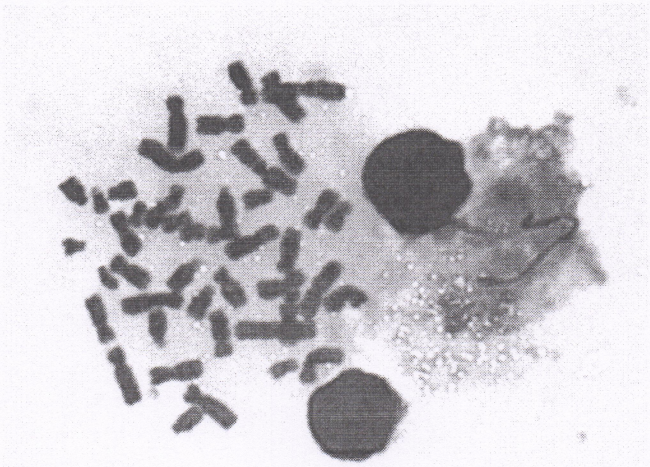
The metaphase arresting solution (MAS) reached the end of its shelf life at the start of the experiments, so even though this reagent produced a greater number of quality spreads per slide, the colcemid remained the mitogen used because the MAS no longer functioned. No spreads were obtained because nothing was arresting the cells in metaphase. Once the MAS was replaced by colcemid, there were a number of spreads on each slide. The other reagents used did not pose any difficulties or compromise the spreads.

The banding of the chromosomes was enhanced from the previous procedure (Werst, 2005). The use of trypsin instead of pancreatin produced clear banding patterns when viewed through a microscope. The images themselves are still not fully optimized, but processing the images obtained in Photoshop helped show that bands are present on the chromosomes and enhanced detail in the image that the camera was unable to fully capture. The culturing and harvesting procedures are complete and, as they stand, produce quality chromosome spreads with banding patterns that are eligible for karyotyping. These images do not translate to those captured with the camera, and thus can not be sent for karyotype analysis. This is mostly due to the fact that the Optronics Magnafire camera is best suited for fluorescence imaging, not light microscopy imaging. The use of a light microscope camera could help enhance the quality of the images.

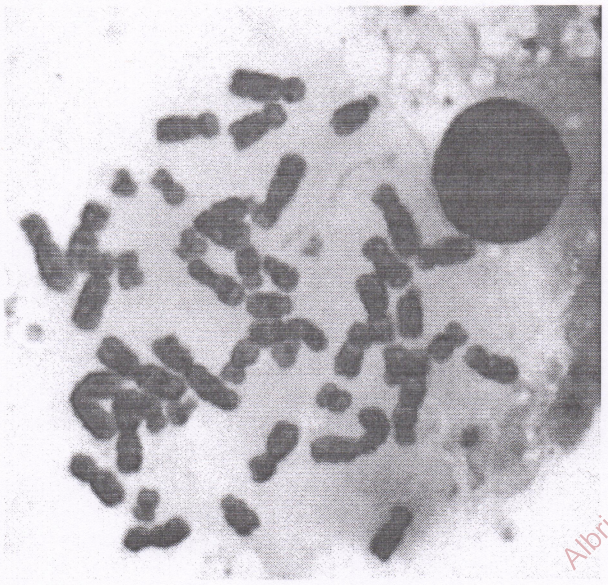
In conclusion, this project is, to the best of our knowledge, the first successful attempt at using low blood volumes of human blood to culture cells, and to ultimately obtain banded chromosome spreads that potentially can yield a karyotype. The imaging could be improved upon, but the use of a camera that is suited to light microscopy imaging should solve the problem of the spread as viewed through a microscope not properly translating into the captured image.

Appendix A

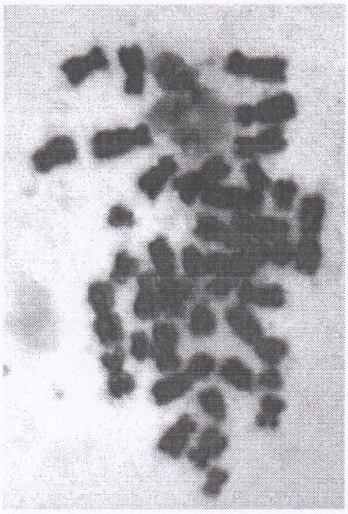
Figure 1: Varied Trypsin Digest Times



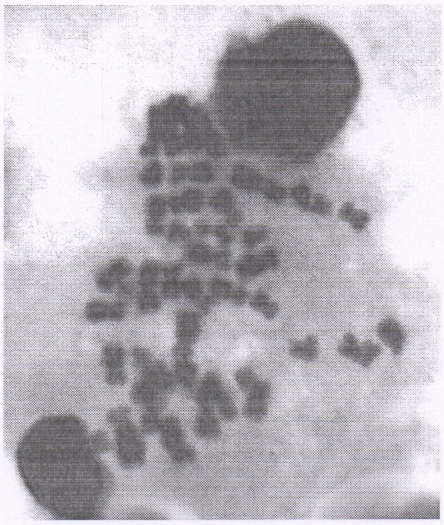
a. This image shows a chromosome spread that was digested with trypsin for 5 seconds before staining. There are no strong banding patterns evident on the chromosomes. The chromosomes are clear and crisp in the image.



b. This image shows a chromosome spread that was digested with trypsin for 8 seconds before staining. The chromosomes are crisp and clear, and bands can be seen on the individual chromosomes. The background of the image, which contains other cells and most likely remnants of the cytoplasm, make this image less than optimal, but the image shows that this digest time produces bands on the chromosomes.

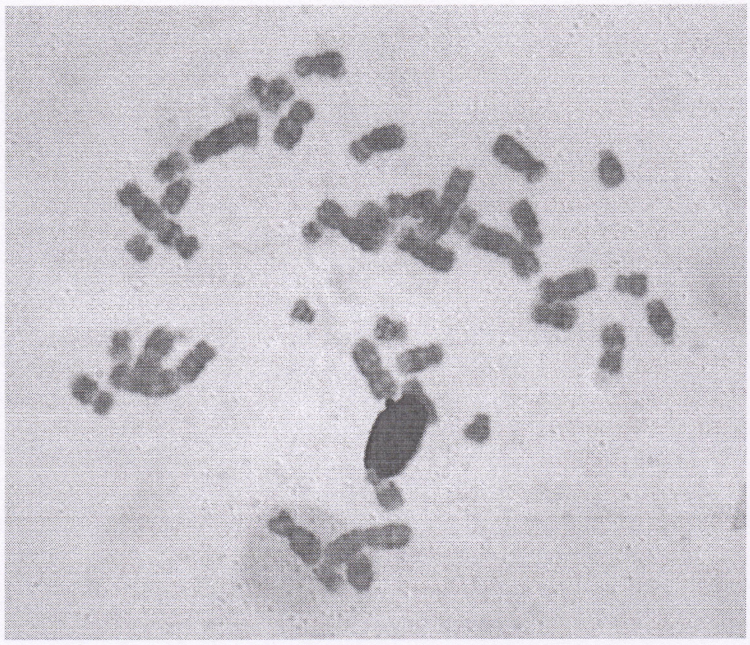


c. This image shows a chromosome spread that was digested with trypsin for 10 seconds prior to staining. The chromosomes are "fuzzy" and the image has an overall hazy appearance. There are no clear bands evident on the individual chromosomes.

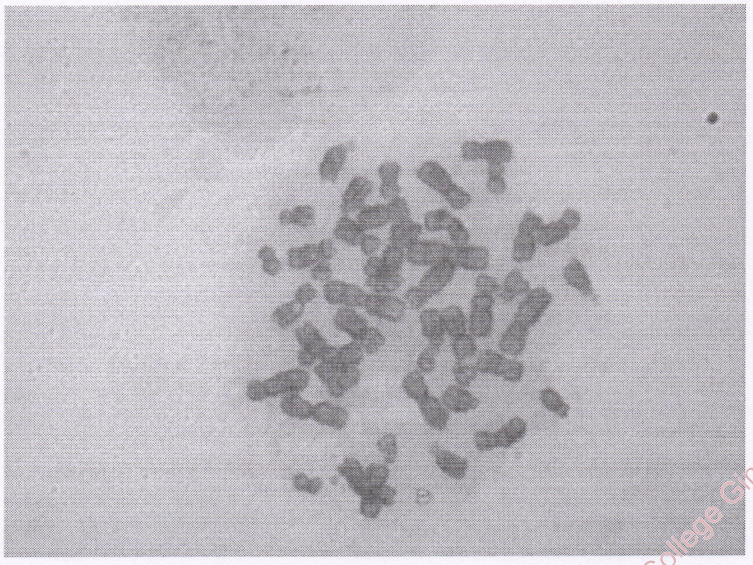


d. This image shows a chromosome spread that was digested with trypsin for 15 seconds prior to staining. The chromosomes are very fuzzy and the image is hazy. No bands are evident on the chromosomes. This spread has been overdigested, which gives the image the hazy appearance.

Figure 2: Example of quality chromosome spreads with banding.



The chromosomes in this image are clear and crisp, with little crossover in the spread. Bands are clear on the individual chromosomes. This type of spread is possibly eligible for karyotyping.



The chromosomes in this image have banding, which the grayscale helps to show better than the color image above.

Appendix B

Karyotyping Consent Form Example

Karyotyping Consent Form

The Cell Biology (Bio 332) class will be culturing white blood cells donated by students in the class for the purpose of karyotyping (chromosome analysis). Blood will be obtained via a sterile pinprick of the fingertip. A spring-loaded pen used for diabetic blood analysis will be loaded with a sterile, hair-thin needle to produce a virtually painless drop of blood. Individual needles will be disposed of in the sharps waste container and anything else in contact with blood will be autoclaved. Participation in this exercise is voluntary and those who opt out may choose to observe others if they wish.

It is possible that you may find out information about yourself during the course of karyotyping that you do not want others to share. For example, you may be an XXX female or an XXY male. In signing this consent form, each person acknowledges that there is the potential for finding out sensitive information about themselves and/or others in the class. This information must be kept confidential. Signing this form means that you will honor confidentiality and behave in a mature fashion if such information is revealed. In other words, should a peer find out something sensitive you will not overreact to the results, you will not announce these results to others within and/or outside of the class, and you will not belittle any person receiving the results.

Check all that apply and sign on the line at the bottom of the page:

_____ I understand that obtaining blood via fingertip prick carries a low level of risk to me, but must follow sterile procedures carefully to ensure the welfare of other participants.

_____ I understand that karyotyping may reveal personal information and I will keep such information confidential.

_____ _____

Signature Date

Appendix C: Laboratory Protocol

Culturing

1. Work in the laminar flow hood, and spray gloves with 70% ethanol.
2. Obtain an Elisa Plate and place 160 µL of karomax medium into one well.
3. Remove gloves and wipe fingertips with alcohol towelette.
4. Remaining in the hood, prick the tip of one or more fingers to obtain approximately 20 µL of blood.
5. The blood can be drawn off the finger(s) using a micropipette and sterile tip.
6. Place blood into the well containing the medium (total volume: 180 µL).
7. Repeat steps 1-6 if additional wells are to be filled.
8. Incubate the culture in the CO_2 incubator at 37°C for 3 to 4 days.

Harvesting

9. 30 minutes prior to adding the colcemid, add 2 µL of chromosome resolution additive (CRA) working solution to each well. To make the working solution add 1 µL of CRA to 99 µL of sterile water and vortex to mix in a centrifuge tube.
10. Two hours prior to harvesting, add 18 µL of Karyomax colcemid solution to each well.
11. Transfer the culture to a 1.5 mL centrifuge tube and spin at 2,000 rpm for 5 minutes.
12. Remove the supernatant and add 200 µL of karomax and 20 µL of pre-warmed pre-hypotonic solution; mix pellet gently by flicking the end of the centrifuge tube.
13. Centrifuge for 10 minutes at 1000 rpm.
14. Remove the supernatant and add 250 µL of pre-warmed optimal hypotonic solution to the centrifuge tube drop by drop, folding the pellet into solution. Be sure to blow the remaining liquid in the tip of the micropipette out into the solution before disposing of the tip.
15. Incubate the solution at 37°C for 20 minutes.
16. During the incubation time, mix the fixative: 1 mL of acetic acid and 3 mL of methanol to a Falcon tube. Add 20 µL of cytoclear. Vortex to mix.
17. After the incubation period, spin the solution at 2,000 rpm for 5 minutes.
18. Remove the supernatant and add 250 µL of the fixative with cytoclear drop by drop to the remaining pellet. As with the hypotonic solution, "fold" the pellet into the fixative solution in order to gently break it apart. Blow out any residual liquid.
19. Let the solution stand with the fixative and cytoclear for 10 minutes.
20. Place slides in 95% ethanol.
21. Centrifuge the solution at 2,000 rpm for 5 minutes.
22. Remove the supernatant and resuspend the pellet as before in the fixative and cytoclear solution.

Dropping

23. Remove the slides from the ethanol and allow the slides to dry.
24. After the slide is dry, place the slide on ice as flat as possible so the majority of the slide is touching the ice.
25. Draw up the fixed cells from the centrifuge tube into a Pasteur pipette.
26. Drop the suspended cells from two foot above the slide. Allow the slides to dry.

Staining

Straight Giemsa Stain

27. Dip the slides in Gurr buffer for 30 seconds, and allow the slides to dry.
28. Cover the slide entirely with 1 mL of Giemsa stain, and allow the stain to stand for 10 minutes.
29. Rinse off the stain with DI water and allow the slides to dry.

G-Banding Stain

1. Place the slides to be stained in an oven (55°-60°C) to age the slides overnight. Allow the slides to come to room temperature before staining.
2. Make up the following working solutions, each in a different coplin jar:
   -Trypsin Solution: Add 1.5 mL of 2.5% trypsin to 50 mL of Hank's Balanced Salt Solution
   -Fetal Bovine Solution: Add 1 mL of FBS to 50 mL of Hank's Balanced Salt Solution
   -Buffer Rinse: 50 mL of Gurr buffer in a coplin jar
   -Giemsa Stain Solution: Add 1 mL of Giemsa stain to 50 mL of Gurr buffer
   -Water Rinse: 50 mL of DI water in a coplin jar
3. Dip each slide, one at a time, in the trypsin solution and move back and forth for 8 seconds.
4. Dip in FBS solution and move back and forth for 5 seconds.
5. Dip in buffer rinse and move back and forth for 5 seconds.
6. Place in Giemsa solution for 9 minutes.
7. Dip in water rinse and move back and forth for 5 seconds.
8. Allow the slides to dry overnight before coverslipping.

Literature Cited

Benn, P.A. and M.A. Perle. Human Cytogenetics: A Practical Approach. 1992. 2nd ed. Eds. D.E. Roomey and B.H. Czepulkowski. IRL Press at Oxford Univ. Press. Vol. 1. p. 91-101.

Cooper, G.M. Cell: A Molecular Approach. 1998. ASM Press. Washington, D.C. pp. 1-673.

Freshney, I.R. Culture of Animal Cells: A Manual of Basic Techniques. 2000. 4th ed. John Wiley & Sons, Inc., Publication. New York. pp. 1-577.

Gosden, C.M., D. Davidson, and M. Robertson. Human Cytogenetics: A Practical Approach. 1992. 2nd ed. Eds. D.E. Roomey and B.H. Czepulkowski. IRL Press at Oxford Univ. Press. Vol. 1. p. 2-81.

Greenwell, I. "Glutamine: The Essential 'Non-Essential' Amino Acid." LE Magazine. 1999. <http://www.lef.org/magazine/mag99/sep99-report3.html>.

Mather, J.P. and P.E. Roberts. Introduction to Cell and Tissue Culture: Tissue and Technique. 1998. Gentech Inc. Plenum Press. New York, NY. pp. 1-241.

Merriam-Webster Online Dictionary. 2005. Merriam-Webster, Inc. <http://www.m-w.com/>.

National Institute of Health, Section of Cancer Genomics, Genetics Branch, NCI. Giemsa-Trypsin Banding. 2005.

Nichols, W.W. and D.G. Murphy. International Review of Cytology: Differentiated Cells in Aging Research. 1979. Academic Press. New York, NY. pp. 1-215.

Prenatal Diagnosis. 2006. <http://www.medlib.med.utah.edu/webpath/TUTORIAL/PRENATAL/PRENATAL.html>.

Rainbow Scientific. 2005. <http://www.rainbowscientific.com/products.html>.

Richardson, A.M. "Chromosome Analysis." The AGT Cytogenetics Laboratory Manual. 1997. 3rd ed. Eds. M.J. Barch, T. Knutsen, and J. Spurbeck. Lippincott-Raven Publishers. p. 481-526.

Saitoh, Y. and U.K. Laemmli. 1994. Metaphase Chromosome Structure: Bands Arise from a Differential Folding Path of the Highly AT-Rich Scaffold. *Cell*. February 25, Cell Press. 76: 609-622.

Werst, L. "Low Volume Human Blood Cell Culture and Karyotyping." 2005. Honors Thesis, Albright College.

Wolstenholme, J. Human Cytogenetics: A Practical Approach. 1992. 2nd ed. Eds. D.E. Roomey and B.H. Czepulkowski. IRL Press at Oxford Univ. Press. Vol. 1. pp. 3-29.