The Genetic Contribution of Sperm: Healthy Baby or Not?

Members of the sperm and embryo cytogenetics team at LLNL. Back row, left to right: Francesco Marchetti, Jiri Rubes, Andy Wyrobek (principal investigator), Joyce deStoppelaar, and Paul Van Hummelen. Front row, left to right: Armand Tcheong, Emily Panico, Xiu Lowe, Nancy Oschbach, and Mike Cassell. Not shown: Christina Sanders, Thomas Ahlborn, Adi Baumgartner, Luoann Uelese, Wendie Robbins, Elizabeth Notley, and Janet Baulch.

We are developing powerful molecular methods to visualize individual chromosomes in sperm and to detect genetic defects in embryos. Our research methods, combined with animal models, have broad implications for screening males with chromosomal abnormalities and genetic diseases, for studying the effects of exposure to mutagenic agents, and for assessing genetic risks to the embryo and offspring.

THE global population explosion would seem to suggest that human reproduction functions quite well. However, reproductive failures, abnormalities during pregnancy, and birth defects are more common than many people realize. Every year in the U.S., more than 2 million couples who want to have children are infertile, and over 2 million conceptions are lost before the twentieth week of gestation. In addition, about 7% of newborns have low birth weight, and up to 7% of babies, or about 210,000 children per year in the U.S., are born with some birth defect. Half of these birth defects are major, affecting the health and viability of the individual.

The social and medical costs of reproductive abnormalities are formidable, yet their causes are not well understood. Abnormal reproductive outcomes include a wide variety of problems listed in Figure 1. Different molecular mechanisms, diagnoses, and treatments are typically involved in the different conditions. The cause of almost any reproductive abnormality can be the result of genetic and physiological events that occurred in any one (or in some combination) of three people—the mother, father, and child. Because of such complexities, pinpointing the cause of a specific reproductive abnormality may be even more difficult than determining the cause of cancer.

Certain abnormal reproductive outcomes can be caused by events that occurred in the germ cells (sperm or egg) of one of the parents before fertilization. Some abnormal reproductive outcomes, such as Down syndrome, have been traced to abnormalities in the eggs of the mother. Historically, the picture has been much less clear for the father. Now, we have compelling evidence that the male parent can be the source of detrimental effects on the genetic makeup and health of the embryo and child.

Geneticists estimate that about 40% of the cases of human infertility are due to male factors. About 80% of chromosomal aberrations (structural defects in chromosomes seen at birth) originate from the father. Furthermore, almost all new gene mutations seen in offspring and most abnormalities in the numbers of the sex chromosomes come from the father's sperm. Nevertheless, we have only had a limited understanding of the details underlying the father's contribution to reproductive problems and failures.

Biomedical scientists at the Laboratory are now conducting research on chromosomal defects in sperm and their effects on the developing embryo. Until recently, little was known about such defects because no practical method was available for detecting abnormal chromosomes in sperm and early embryos.

Three Kinds of Evidence

Three primary lines of evidence form the basis for our research.

Declining Sperm Count

First, the sperm count of men has been declining over the last five decades, and we still do not know exactly what accounts for the decline. In 1983, we conducted studies for the U.S. Environmental Protection Agency (EPA) on the effects of nearly one hundred different types of exposures on sperm production in human males.[1] About half of the agents we studied, including alcoholic beverages, cigarette smoke, and lead, lowered the production of sperm or affected sperm motility or morphology.

Occupational Exposure

A second important line of evidence suggests that certain jobs and workplace and environmental exposures of the father are linked to spontaneous abortion and problems in their offspring, including birth defects and cancer. Some occupations seem to be repeatedly associated with abnormal reproductive outcomes; however, findings are variable, and actual exposures are often poorly defined. We still do not have conclusive links between specific exposures, mechanisms of transmission, and an increased frequency of birth defects or childhood cancers.

At least two different models can account for many of the epidemiological findings. It is possible that some fathers might bring home potentially damaging agents on equipment, skin, or clothing, thus affecting the wife, offspring, or both. On the other hand, the route of exposure could be more direct, via the father's sperm. LLNL researchers have been developing and applying improved sperm assays to help distinguish between these two models.

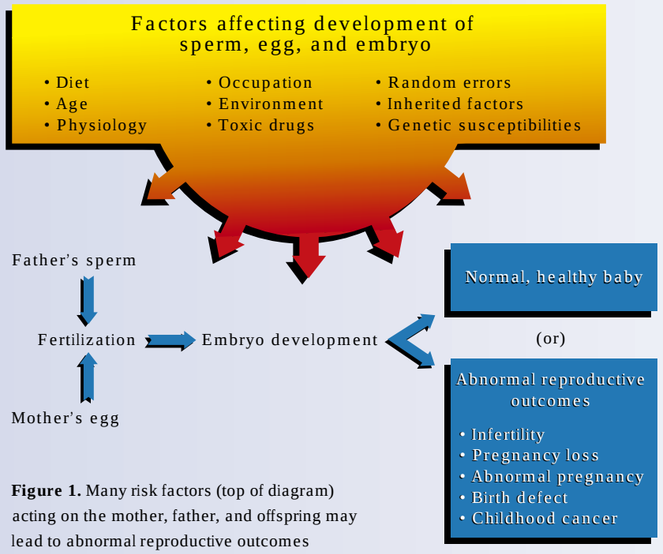


Figure 1. Many risk factors (top of diagram) acting on the mother, father, and offspring may lead to abnormal reproductive outcomes (bottom, right). Some risks can date to shortly after the time of conception of either parent. Livermore researchers are focusing on defects in sperm that lead to abnormal outcomes.

Genetic Mutations in Infants

A third line of evidence for male-mediated reproductive effects comes from studies of babies when some of the newest molecular methods are applied. Such studies show that when entirely new gene mutations occur in an offspring—ones never seen before in either the mother's or the father's family—they are almost always associated with the father's genes. Several defects also predominantly occur in the father's chromosomes. For example, about 80% of the chromosomal aberrations seen in the chromosomes of babies—defects such as chromosome breaks—come from the father.

Confusing Evidence

For decades, we have known that male animals—especially laboratory mice and rats—that are exposed to certain damaging agents can suffer adverse reproductive effects and other

Science & Technology Review November/December 1995

health problems. The effects can include reduced sperm production, diminished quality of sperm, and reduced libido.

Researchers can systematically study rodents to gain a better understanding of the links between exposure and reproductive effects. However, for humans, we must rely on the few sources of evidence that are available to us, including exposed individuals and their offspring. Studies since the 1950s have consistently shown that exposures of human males to environmental, occupational, or therapeutic agents can have detrimental effects on sperm count, motion, or shape. In contrast, although many environmental agents clearly have mutagenic potential in animals, experts have disagreed on whether environmental exposure of human males contributes very much to genetic disease or to adverse effects in their offspring.

For example, the research literature is consistent in confirming the adverse genetic effects of ionizing radiation in the male mouse and its offspring. However, the human offspring of atom-bomb survivors have no measurable increase in induced mutations. Similarly, exposures to various agents used in chemotherapy and to radiation therapy do not yield clear-cut results for genetic effects in the offspring of treated male patients.

Some of the puzzling inconsistencies between humans and mice may be due to individual variation and species differences. Other explanations involve the role of DNA repair processes or the possibility that some chemical or physical agents (mutagens) may have limited or short-term effects on sperm. Human doses are often small compared to those used in research on mice, and the number of human offspring that have been studied for induced genetic effects remains relatively small. Finally, it is possible that the types of genetic damage (called "endpoints" by geneticists) assessed in studies of exposed humans are not sensitive enough to always reveal a significant effect.

A Review of the Basics

The body (or somatic) cells of humans and other mammals contain pairs of chromosomes. Except for the sperm or egg cells and red blood cells, human somatic cells carry 46 chromosomes (the diploid number). Normal human somatic cells have 22 pairs of autosomes (nonsex chromosomes) and one pair of sex chromosomes, either XX or XY. Of the sex chromosomes, a normal female carries two X chromosomes, and a normal male carries one X and one Y chromosome.

(a) Normal human sperm carrying 22 autosomes and a Y chromosome.

(b) Normal human sperm carrying 22 autosomes and an X chromosome.

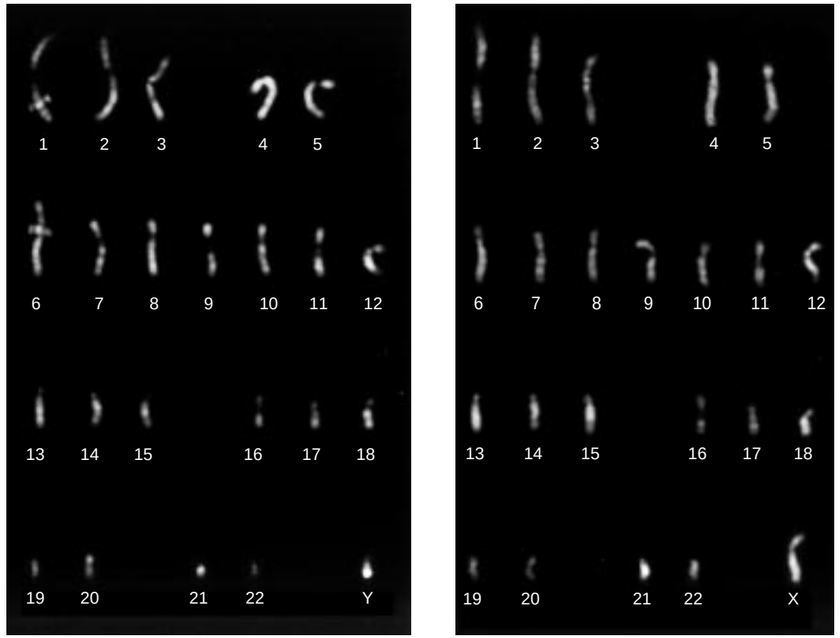


Figure 2. Normal human somatic cells carry 46 chromosomes, but each sperm and egg carries only 23 chromosomes (the haploid number). The photomicrographs show the normal complement of 23 chromosomes from two human sperm that were specially prepared to make the chromosomes visible under a microscope (ref. 4). One sperm (a) carries the Y chromosome and would produce a male; the other (b) carries the X chromosome and would produce a female. (Photographs courtesy of L. Gordon and B. Brandriff of LLNL.)

In contrast to somatic cells, each sperm and egg contains 23 chromosomes (the haploid number in humans). Each normal sperm and egg carries one copy of chromosome 1, one copy of chromosome 2, and so forth. Figure 2 shows the haploid number of chromosomes from human sperm that were specially prepared by a technique developed at the Laboratory.

If either of the germ cells carries an abnormal number of chromosomes or some other genetic defect, major hazards may arise for the offspring. A fetus resulting, for example, from fertilization with a genetically defective sperm would carry a mutation not only in the germ tissues but also in all somatic cells. An embryo's survival and quality of life through birth and beyond depend on the specific chromosomal defect it may carry. An embryo carrying major chromosomal defects will die during development. Thus, a validated method for detecting chromosome abnormalities in sperm has broad implications for maintaining or improving human health.

About Aneuploidy

The measure (or biological marker of male reproductive risk) we have chosen to study in depth is sperm aneuploidy. Aneuploidies in general are an important category of chromosomal damage that can be transmitted to an offspring from either the father or mother. The word "aneuploidy" refers to cells carrying the wrong (thus the prefix "an") number of chromosomes ("euploid"). Aneuploidy is one of the most common and serious chromosomal abnormalities recognized in humans. It is responsible for a large portion of infertility, pregnancy loss, infant death, malformations, mental retardation, and behavioral abnormalities.

Human embryos with an abnormal number of sex chromosomes or with an extra chromosome 13, 18, or 21 can survive to birth and beyond. An extra chromosome 21 causes Down syndrome and is a familiar example of aneuploidy involving one of the nonsex chromosomes. However, the most common aneuploidies in humans at birth involve an abnormal number of X or Y chromosomes. This condition, sex-chromosome aneuploidy, can be diagnosed prenatally through amniocentesis, and the incidence is about 1 in 250.

Table 1 shows different types of sex-chromosome aneuploidies together with other abnormalities involving the autosomes. A male child who inherits, say, an extra Y chromosome from the father would have a total of 47 chromosomes and a sex-chromosome aneuploidy (XYY). We know that human fathers are responsible for 100% of 47, XYY cases because the mother carries no Y chromosome. Another aneuploidy involving the sex chromosomes is Turner syndrome (45, XO), in which a paternal chromosome is lacking about 80% of the time. Other conditions are Klinefelter syndrome (47, XXY) and a triplet of X chromosomes (47, XXX).

Slightly more than half of the sex-chromosome aneuploidies at birth

Table 1. Examples of aneuploidy in the sperm of humans. Abnormal chromosomal conditions arise in the embryo when a sperm contributes an abnormal number of chromosomes to the embryo. The normal complement of sex chromosomes is shown in the shaded area at the top for comparison.

Father's contribution in sperm	Mother's contribution in egg	Embryo or offspring	Syndrome
X	X	46,XX	Normal female
Y	X	46,XY	Normal male
XY	X	47,XXY	Klinefelter syndrome • Hypogonadism • Sterile
No sex chromosome	X	45,XO	Turner syndrome • Characteristic physical features • Hypogonadism • Sterile
YY	X	47,XYY	XYY male
XX	X	47,XXX	XXX female
21,21	21	47,+21*	Down syndrome • Mental retardation • Characteristic physical features
18,18	18	47,+18*	Edward syndrome • Mental deficiency • Anomalous hands, face • Often fatal
13,13	13	47,+13*	Trisomy 13 • Severe anomalies • Often fatal

*The extra autosome is often contributed by an aneuploid egg, but sperm are also known to be responsible for the genetic defect.

are of paternal origin. The effects of such aneuploidy depend on which combination of X and Y chromosomes is involved. The health effects of XYY, for example, are minor; however, the effects of Turner and Klinefelter syndrome include physical, behavioral, and intellectual impairment as well as sterility.

Among human babies, the frequency of known chromosomal abnormalities, including aneuploidies and structural aberrations, is about 0.6%. In addition, about 1% of newborns carry a mutation for a genetic disease.[2] When an inherited error occurs, we need some way to ascertain when the condition is due to the father, what factors can cause the condition, and what prevention strategies might be effective.

Assessing Damage

The DNA complex forming the chromosomes (chromatin) in sperm is typically rigid and so dense that it occupies nearly the minimum possible volume. The high degree of condensation makes it nearly impossible to visualize and identify individual chromosomes by standard light or electron microscopies. LLNL researchers have been involved in the development of several techniques that allow sperm chromosomes to be visualized and assessed for anomalies.

The Hamster Technique

In 1978, Rudak and colleagues working in Hawaii pioneered a way to analyze the chromosomes in human sperm after fusion with hamster eggs.[3] Until recently, this "hamster technique" was the only method available for characterizing chromosomal defects in human sperm.

During the 1980s, LLNL researchers were the first to get the hamster technique to reliably work for a variety of applications, a considerable challenge because biologists elsewhere in the world were having major difficulties in obtaining usable results. (The March 1984 issue of *Energy and Technology Review* provides a more complete description of this highly useful tool.)[4] We showed that the hamster technique gives valuable baseline information on the normal burden of damage in healthy men. Using the method, we found that a small proportion of sperm in otherwise healthy males carries aneuploidies or other types of structural aberrations.

The hamster technique has become a highly reliable tool, and we consider it to be the "gold standard" against which we evaluate any new methods. However, the technique is difficult to apply and is both labor-intensive and inefficient, so it is costly to perform.

Fluorescence *In Situ* Hybridization

By about 1990, the Laboratory had developed a new biological procedure that could detect aneuploid sperm more efficiently than the hamster technique. Fluorescence *in situ* hybridization (FISH) has been previously described in *Energy and Technology Review* (see the April/May 1992 issue) as a gene-mapping tool.[5] The method is illustrated in the context of our sperm research in the box on page 12. In essence, we prepare chemically labeled DNA probes and bind (hybridize) them to target chromosomal DNA within the sperm head.

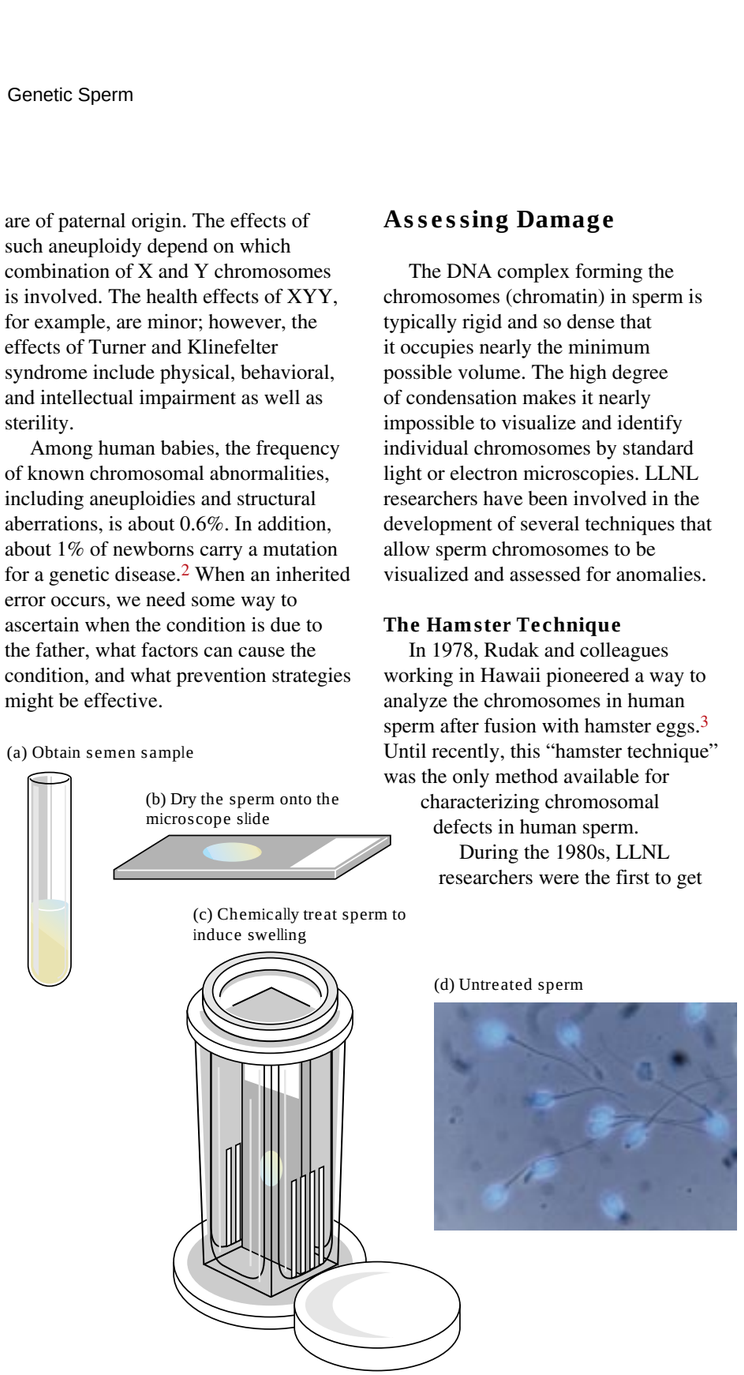


Figure 3. The high degree of condensation of the DNA–protein complex (called chromatin) in sperm makes it nearly impossible to identify individual chromosomes by standard microscopy. The two photos show how our chemical treatments (a–c) swell the sperm head, which is shown before (d) and after (e) treatment. The scale is the same in (d) and (e).

One of the challenges we met in applying FISH to human sperm was finding the right chemical treatments needed for introducing probes with fluorescent tags into the dense sperm head to penetrate the DNA. We learned how to control the amount of swelling (see Figure 3) that occurs during the process while maintaining the integrity of the nuclear material in sperm. FISH is especially useful because it provides vivid fluorescent signals, it allows us to distinguish between several probes with different colors, and it is reliable.

First Use of FISH in Sperm

In our first demonstration of FISH in human sperm, we applied a fluorescently labeled DNA probe to the Y chromosomes of sperm from human volunteers. As shown in Figure 4a, the Y chromosomes were tagged with a fluorescein label, a green-fluorescing dye that can look yellow, for example, on a red background. The Y chromosomes are easily recognized as bright yellow spots, called "domains." We counterstained the sperm nuclei with the red-fluorescing dye propidium iodide, which produces the bright red background color.

After examining and scoring 11,500 sperm nuclei, we found that 50% of sperm showed fluorescent domains consistent with the presence of a Y chromosome. The proportion is what we would expect, because about half of all sperm carry a Y chromosome, and half carry an X chromosome. This finding is also consistent with the proportion of sperm containing Y chromosomes as determined by the hamster technique. As anticipated, FISH proved to be a direct and reproducible method for monitoring the chromosome constitution of sperm, and it allows us to visually analyze thousands of cells rapidly. In subsequent studies, we expanded the number of DNA probes we can apply to sperm nuclei, allowing us to tag two or three different chromosomes simultaneously, and we extended the method for use in laboratory animals.

Extending the Human Assay

Two-Probe Assays

Because aneuploidy at birth frequently involves the two sex chromosomes, we initially extended the FISH assay to include the second sex chromosome (chromosome X). Figure 4b shows normal sperm carrying either a single X chromosome, which fluoresces blue-green due to the dye FITC, or a single Y chromosome, which fluoresces red due to the dye Texas Red. In the photograph, the red domains are larger than the blue-green ones because the DNA regions we targeted on the Y chromosome had longer repetitive sequences. In this and subsequent photos, the precise color of a fluorescent dye can vary as a function of counterstains used to highlight the sperm nucleus.

Beyond studies of normal sperm, the two-probe assay gives us a method for detecting sperm carrying an abnormal number of chromosomes X and Y. This type of assay can be applied to study sperm that give rise to Turner syndrome, Klinefelter syndrome, and other inherited sex-chromosome conditions. When such aneuploid sperm are produced, our two-probe assay can differentiate among sperm containing two red domains (YY), two green domains (XX), or both colors (XY).

Three-Probe Assays

Next, we added a fluorescently labeled DNA probe for one of the autosomes in sperm. Whereas any autosome would suit the purpose, we selected a probe for chromosome 8, which was our best DNA probe available at the time. Adding one autosome to FISH is a major advantage because it allows us to distinguish among three possibilities: duplication of a sex chromosome only (sex-chromosome aneuploidy), duplication of a single autosome only (autosomal aneuploidy), and duplication of the

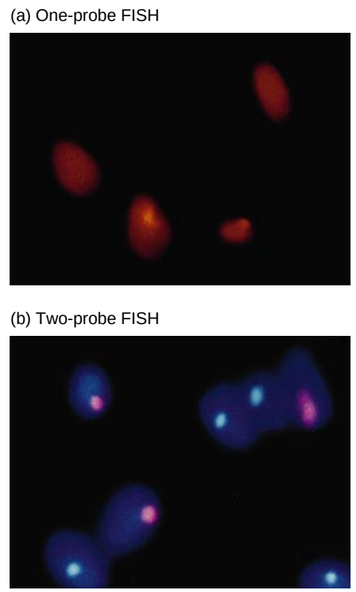


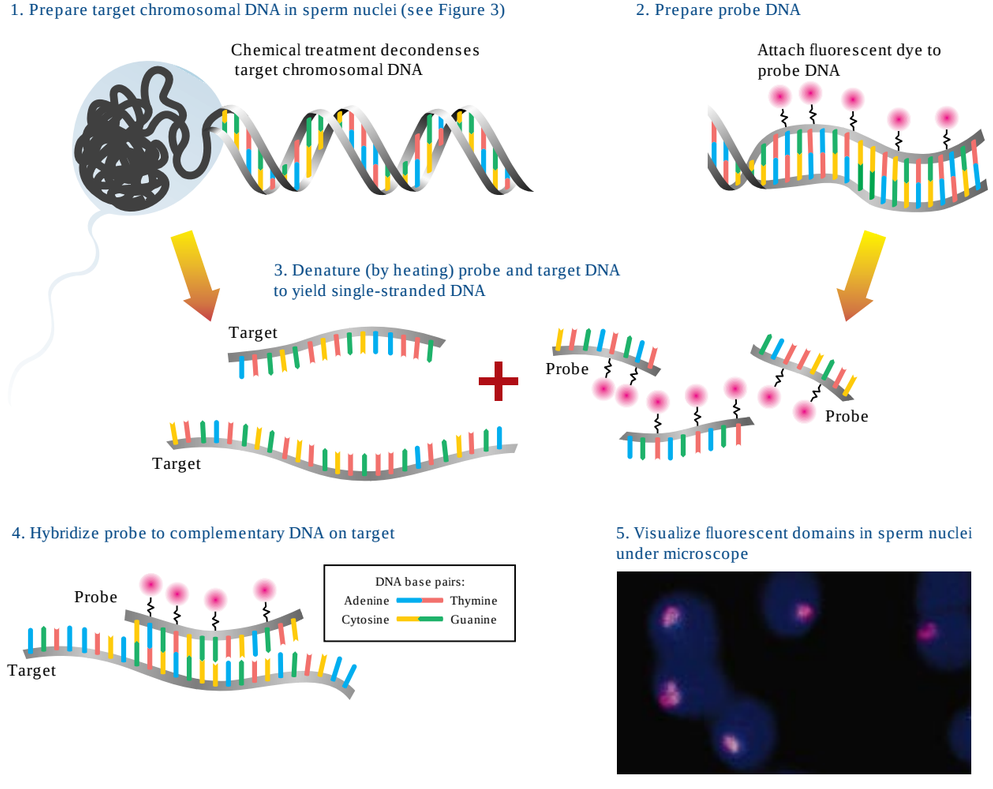
Figure 4. (a) The fluorescent dye fluorescein is applied to the Y chromosomes in human sperm. When they are present (50% of the time), the Y chromosomes are easily recognized as bright yellow areas. The sperm nuclei are counterstained with propidium iodide, giving a red background color. (b) This example of our two-probe FISH procedure shows how we can differentiate among human sperm carrying a single Y chromosome (red fluorescence) or a single X chromosome (green fluorescence).

How FISH Is Used to Detect Aneuploid Sperm

Abnormal human sperm can contain two X chromosomes, two Y chromosomes, neither X or Y, both X and Y, and an abnormal number of autosomes (either more or less than 22). How can we tell the difference between normal and defective sperm when chromosomes are packaged so tightly in the sperm head?

Our technique, called fluorescence *in situ* hybridization (FISH), uses two starting DNA materials: target sperm chromosomes and probe DNA. Sperm carrying the target chromosomes are placed on glass slides. The sperm chromatin is chemically treated, or in technical terms, decondensed, so that our probe DNA can penetrate the chromatin to reach the target chromosomes. The probe consists of DNA fragments (hundreds of copies of a specific region of a particular chromosome) prepared by attaching a fluorescent dye and heating to yield single-stranded DNA. At Livermore, we have DNA probes for most of the human chromosomes, including X and Y, and for many rodent chromosomes. We use several different dyes to differentiate among different chromosome types. When the labeled probes hybridize (bind) with the complementary single strand of target sperm chromosomal DNA, the dyes vividly "light up" the specific region of the chromosome under investigation. We then count and record (score) the fluorescent spots, called domains, which appear as vivid signals through a light microscope.

Our methods are equally successful in studies of human and rodent sperm, and they are far more efficient and less costly than any other assay developed to date, including the hamster technique. Ten thousand cells can be scored in less than two days.



entire genome (diploidy). In other words, if we detected the condition XX88 or YY88, then it is highly likely that all the other chromosomes are duplicated as well (the diploid condition).

Figure 5 illustrates our three-probe assay for chromosomes 8 (yellow), X (green), and Y (red). Figure 5a shows normal human sperm, which carry either X8 or Y8. All such normal sperm fluoresce in only two colors and show two domains (two discrete fluorescent areas). Abnormal human sperm in Figures 5b through 5j show more than two domains (for example, XX88 has four domains) or more than two colors (for example, XY8 has three colors and three domains).

As more FISH probes become available, we will add them to the assay. As soon as an excellent probe for chromosome 21 is developed, we will include it in the FISH assay so that we can look for this important marker for sperm that may lead to Down's syndrome. Similarly, we will soon add DNA probes for chromosomes 13 and 18 because these trisomies, like chromosome 21, survive to birth and beyond in humans.

Recently, we developed another useful tool that has important implications. By adding the technique of phase-contrast imaging to fluorescence microscopy, we can now detect the tails of sperm and distinguish them from somatic cells that are normally present in semen (Figure 6). Differentiation is critical when we detect sperm carrying XY88, for example. Such an arrangement could represent either a diploid sperm or a normal somatic cell (which always carries two sex chromosomes and copies of each autosome). Phase contrast allows us to differentiate clearly between the two possibilities because somatic cells have no tail.

Validating the Method

To demonstrate the utility of the FISH method for assessing sperm chromosomes in humans, we needed to address the issue of validation. How would we know whether the values we obtain—for example, the baseline frequency of aneuploid sperm in healthy males—are actually correct? Fortunately, several lines of evidence from independent sources can be used to validate our assay.

Researchers at Livermore Laboratory and in Canada and Japan have used the hamster technique to collect baseline information establishing the normal

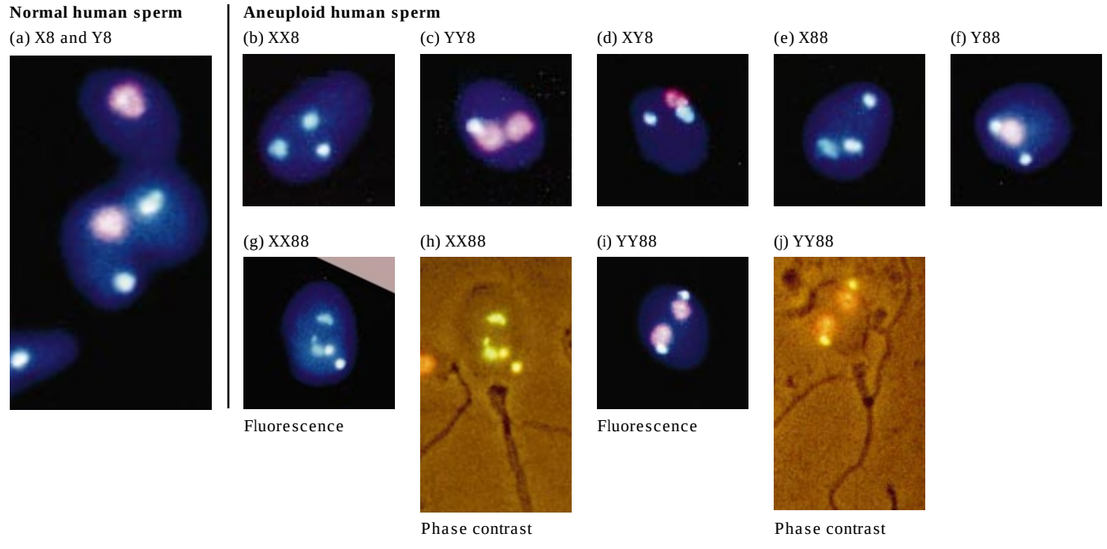


Figure 5. Our three-probe FISH procedure applies a mixture of probes specific for chromosomes X, Y, and 8, each tagged with a different fluorescent dye. Here, human chromosomes X fluoresce green, Y fluoresce red, and 8 fluoresce yellow. (a) Normal sperm carry either X8 or Y8 and are marked by only two different colors and two domains. (b–f) Abnormal sperm, such as XX8, YY8, XY8, X88, or Y88, have three domains but of varying colors. (g–j) Abnormal sperm, such as XX88 or YY88, have two colors but four domains. (h) and (j) show the sperm tail using phase-contrast imaging.

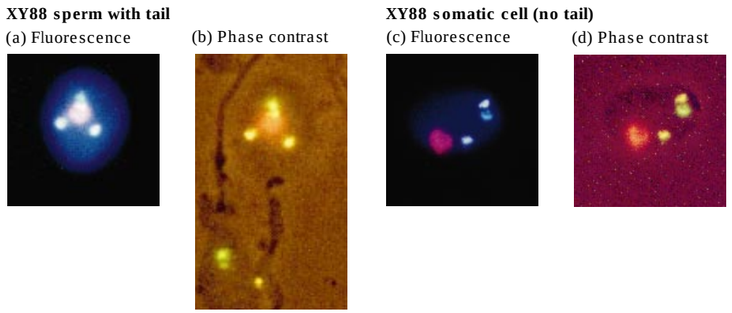


Figure 6. Phase-contrast microscopy, together with fluorescence microscopy, allows us to differentiate between (a and b) XY88 sperm, which have tails, and (c and d) XY88 somatic cells normally present in semen, which do not have tails.

burden of chromosome damage in human sperm. These studies suggest that the baseline frequency of aneuploid sperm in young, healthy males is 3 per 10,000 chromosomes. This is the reference value we used in assessing the new FISH assay. We also have hamster data on the frequency of abnormal chromosomes after administering doses of some mutagenic drugs. Finally, hospitals publish the results of population-based surveys that provide additional statistics on the frequency of XYY babies, XXY babies, and other genetic anomalies at birth.

Baseline FISH Research

Using the one-, two-, and three-probe FISH assays in human sperm, we have assessed chromosomes X, Y, 1, and 8 for evidence of aneuploidy in hundreds of thousands of cells from healthy men. The frequencies of aneuploid sperm can vary among the different chromosome types and among individual male donors. Furthermore, most healthy men give consistent results over time (up to four years).

Our assays show that human sperm contain the abnormal chromosome pairs 8-8, XX, YY, and XY at frequencies of roughly one per 2,000 sperm analyzed, averaging across donors. This frequency is quite similar to the value of 0.6 per 2,000 sperm obtained by the hamster technique. We found that the abnormal chromosome pairs 1-1 had the highest frequency of all, about 3 per 2,000 sperm. No sperm of the many thousands we tested from different healthy donors contains more than two of the same chromosome type (for example, we do not find the triplets 888, 111, or YYY). The most common sex chromosomal abnormality we found using the three-probe assay was XY8, with an average frequency of 9.5 per 10,000 human sperm scored.[6] Table 2 summarizes the frequencies of abnormal sperm types we found in various studies of young, healthy, human males using FISH.

Table 2. Frequencies of human sperm, per 10,000 cells analyzed, with abnormal numbers of chromosomes determined by the FISH method. This summary table represents data from 14 donors and more than 220,000 scored cells. (Remember: normal sperm carry a single X or a single Y and a single copy of each numbered chromosome. Any other combination constitutes a chromosomal abnormality.)

Type of FISH assay	Type of chromosomal abnormality in sperm	Frequency (pooled data for donors studied)
One probe	1-1	14.0
Two probe	YY	5.7
Two probe	XX	3.9
Two probe	XY	6.2
Three probe	XX8	3.1
Three probe	YY8	3.1
Three probe	XY8	9.5
Three probe	88X	3.0
Three probe	88Y	3.6
Three probe	XX88	2.2
Three probe	YY88	1.7
Three probe	XY88	10.6*

*This frequency may be elevated because it may include a small number of somatic cells of the type XY88 normally found in semen.

Bridging Biomarkers

We recently developed corollary methods for detecting aneuploidy in the sperm of mice and rats using two- and three-probe FISH. We used a multicolor FISH procedure to evaluate chromosomes X and 8 in more than 80,000 sperm from healthy, young adult mice. About 3 sperm per 10,000 cells evaluated showed XX or 88 aneuploidies (Figure 7).[7] The frequencies we found for these particular numerical errors in two strains of mice were indistinguishable from those for sperm from healthy men using similar procedures and scoring methods. This work serves to demonstrate what we call "bridging biomarkers" between humans and animals for detecting sperm aneuploidy.

Bridging biomarkers, in essence, allow us to use the same type of measurable variation as we assess similarities and differences among species. With this type of information, we can compare the mean error rate of specific chromosomal defects in human versus rodent sperm, especially the sperm of mice. Our data show that the mean error rate is about the same in otherwise healthy male mice and humans. The bridging biomarkers of sperm aneuploidy also allow us to compare human and laboratory species for effects of physiological changes (e.g., diet, age), effects of exposure to toxicants, and effects of genetic differences. The studies of age effects are summarized to illustrate the utility of bridging biomarkers.

Age Effects in Mice and Men

We divided the 14 men in our three-probe study into two groups with average ages of 47 versus 29 years. Compared to the younger men, older men had higher fractions of abnormal sperm with either two copies of the X chromosome (XX8) or two copies of the Y chromosome (YY8). However, older men did not have higher frequencies of the other possible aneuploid conditions, such as XY8, X88, XY88, and so forth.[6]

Our findings on age effects are preliminary and should be interpreted with caution. A more detailed study using a larger population of men is needed. We have recently received funding from the National Institutes of Health to carry out such a study. The new collaboration will be one of the first human tests potentially linking chromosomally abnormal sperm to age and other life-style factors of the father.

Our preliminary findings of age effects in men are strikingly similar to our recent results on aneuploid sperm in aged mice. Aged mice (mice normally live for about two and a half years) had higher levels of sex-chromosome aneuploidy in sperm than did young mice. Mice of advanced age (older than about two years) had about twice as many aneuploidies of the types XX8, YY8, 88X, and 88Y than did younger mice (slightly older than two months). As with human males, we found the largest age-related increases in the XX8 and YY8 aneuploidies (Figure 8).

If our findings on aging continue to hold up with further research, they may point to an intriguing possibility. According to several lines of evidence on the production of sperm and egg cells with genetic errors, age effects in human females are predominant in the first stage of meiosis (the first of a two-stage process in forming an egg or sperm). Our preliminary data on both mice and human males suggest that age effects in males are predominant at the second stage of meiosis. Thus, males and females may differ in terms of the exact stage at which some genetic errors, such as aneuploidy, arise.

Effects of Smoking

Cigarette smoking is one of the most pervasive examples of the self-administration of toxic compounds. Research over several decades at Livermore and elsewhere has shown that cigarette smoking can cause defects in sperm quality. However, no information has been available on its mutagenic potential in sperm.

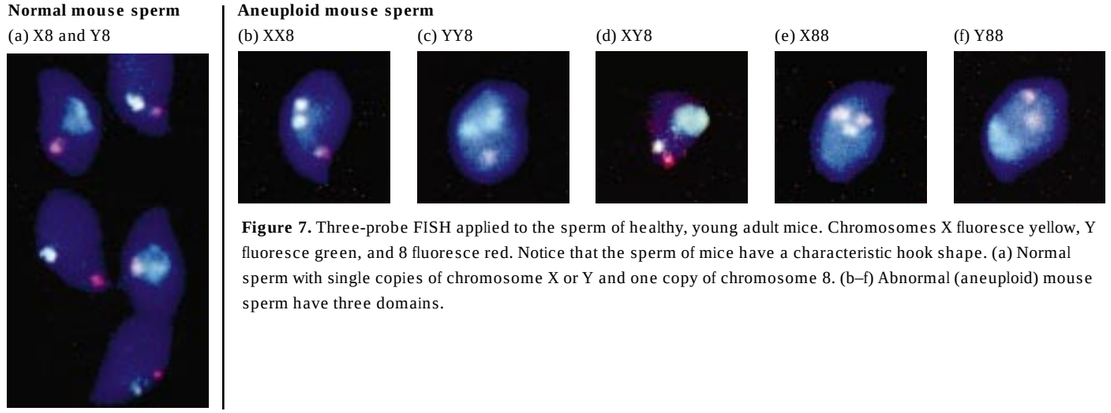


Figure 7. Three-probe FISH applied to the sperm of healthy, young adult mice. Chromosomes X fluoresce yellow, Y fluoresce green, and 8 fluoresce red. Notice that the sperm of mice have a characteristic hook shape. (a) Normal sperm with single copies of chromosome X or Y and one copy of chromosome 8. (b–f) Abnormal (aneuploid) mouse sperm have three domains.

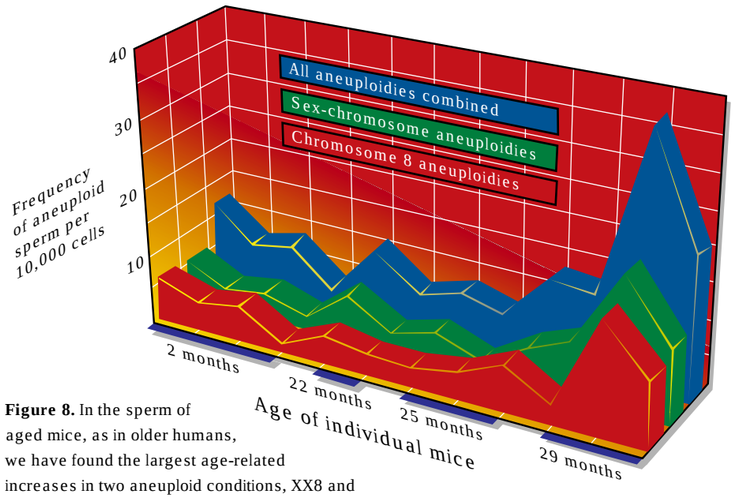


Figure 8. In the sperm of aged mice, as in older humans, we have found the largest age-related increases in two aneuploid conditions, XX8 and YY8. These curves show that the frequencies of aneuploid chromosomes in the sperm of mice increase with increasing age, especially after two years. This figure includes only those instances in which cells have gained a chromosome (ref. 7).

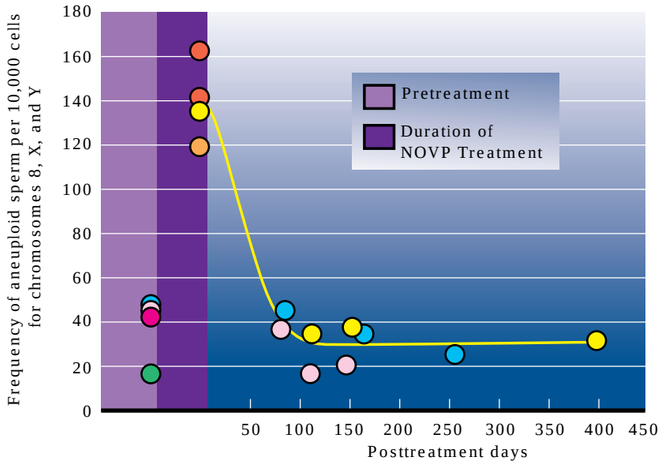


Figure 9. Frequency of aneuploid sperm in young men with Hodgkin's disease undergoing NOVP chemotherapy. The baseline levels of abnormal sperm increase twofold to fivefold following treatment with NOVP and then return to pretreatment levels approximately three months after chemotherapy. Each color represents samples obtained from a separate donor (ref. 6).

We recently studied 15 smokers and 15 nonsmokers from the Czech Republic and found that smokers produce approximately twice the number of aneuploid sperm as nonsmokers. Cigarette smoking is a life-style that often includes alcohol consumption and possible stress factors. Thus, further research is needed to determine whether the effects we found are indeed due to tobacco products or to other aspects of a smoker's life-style.

Effects of Chemotherapy

We have applied the three-probe FISH method to sperm cells of cancer patients before, during, and after treatment with the combination chemotherapy NOVP. Our basic question was whether the aneuploidies induced in sperm might persist following treatment, raising the possibility that genetic damage could be passed on to future offspring. We elected to study NOVP treatment because it contains drugs known to produce aneuploidy in model systems.

Figure 9 shows our results for young male patients with Hodgkin's disease (a kind of lymphatic cancer). When compared with healthy controls, these patients had elevated frequencies of aneuploid chromosomes X, Y, and 8 even before treatment (twofold to sixfold increases over normal levels). Just after NOVP treatment, the frequencies of numerical abnormalities increased twofold to fivefold compared to pretreatment levels. Following chemotherapy, aneuploid sperm returned to pretreatment levels within two to six months—clear evidence that at least one type of chemotherapy has transient effects on aneuploidy in human sperm. Further studies are needed to determine whether other drugs induce aneuploidy in human sperm and whether the effects are also transient.

New Research on Embryos

In very recent work, we have begun to assess damage in embryos, using the mouse as the model species. Figure 10 summarizes the various developmental stages of the mouse embryo at which we can now apply the FISH method to assess numerical and other errors in chromosomes. For example, we can study the embryos immediately after fertilization (just before first cleavage of the embryo when it divides into two cells), at the two-cell stage, or about four days later just before the embryo implants into the uterine wall and when it consists of 30 to 50 cells. This work has required the development of special FISH probes and techniques that are suitable for embryos.

One of the advantages of studying one-cell mouse embryos (called "zygotes") is that the chromosomes from the paternal and maternal mice

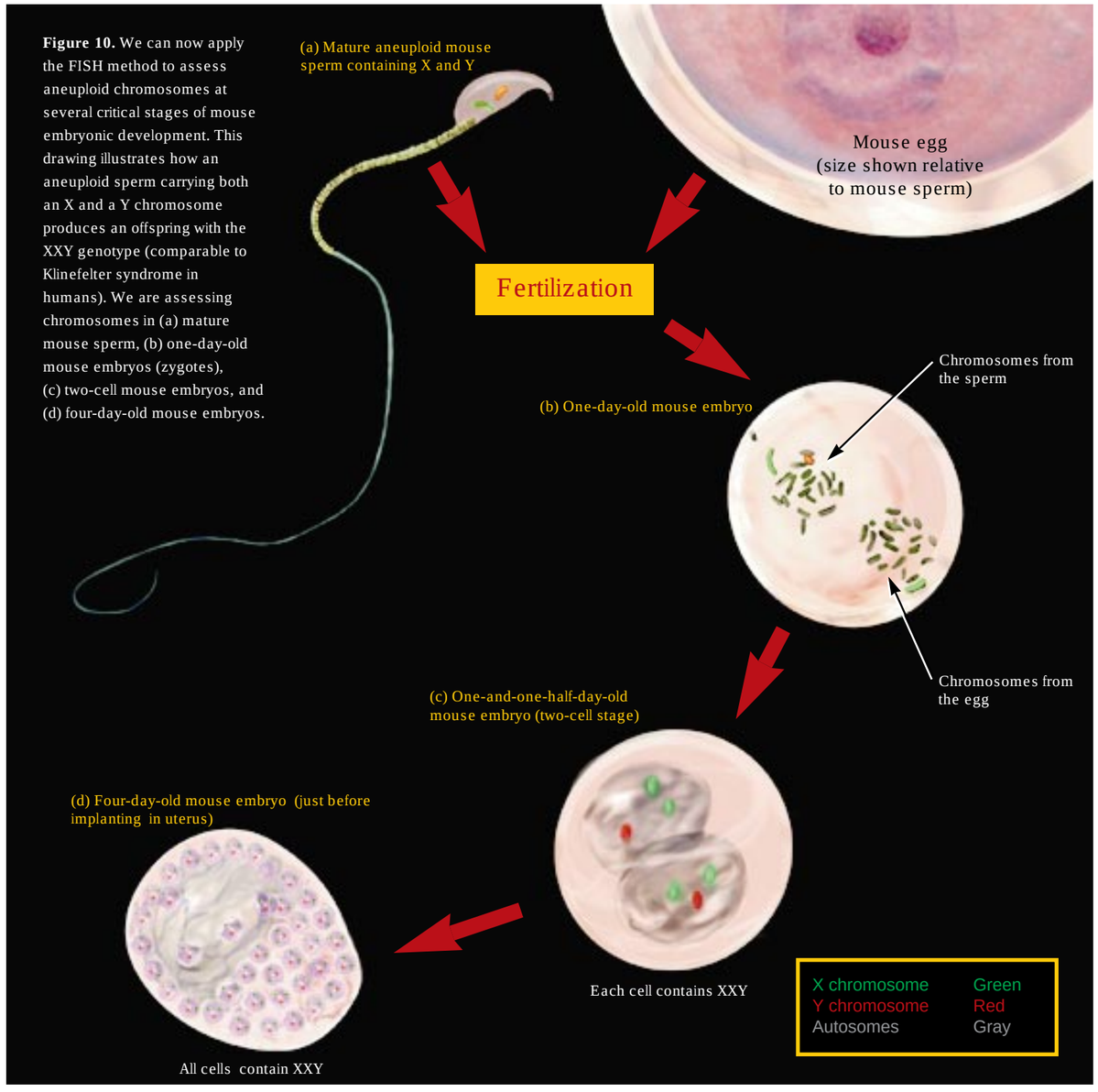


Figure 10. We can now apply the FISH method to assess aneuploid chromosomes at several critical stages of mouse embryonic development. This drawing illustrates how an aneuploid sperm carrying both an X and a Y chromosome produces an offspring with the XXY genotype (comparable to Klinefelter syndrome in humans). We are assessing chromosomes in (a) mature mouse sperm, (b) one-day-old mouse embryos (zygotes), (c) two-cell mouse embryos, and (d) four-day-old mouse embryos.

remain separated until the first cellular division (Figures 11a through 11c). This means that we can determine the contribution of each parent independently. Another advantage is that we can easily validate our results on mice because investigators have published data on the frequencies of aneuploid mouse chromosomes at the time of first cleavage.

Using FISH probes for the Y chromosome, our early results from more than 200 mouse zygotes show that about 9% of the embryos have numerical errors, and two of those cases were of paternal origin (see Figure 11b). We will investigate the effects on the embryo of exposing mouse sperm to chemical agents known to cause genetic defects.

Our new studies on four-day mouse embryos (Figures 11d and e) are a collaboration with the University of California at Berkeley. Very little work has been done on such embryos, so our research will be among the first. We are applying FISH and other biological imaging methods to understand how the development and survival of implanted embryos are affected by mutagen exposure of the father's sperm before mating.

Acrylamide is the model mutagen for our four-day embryo project because it is known to induce heritable defects. The damage we are seeing in embryos includes aneuploidy, mosaics (a combination of some normal cells and some chromosomally altered cells), chromosome breakage, and polyploidy (the occurrence of chromosomes that are three or more times the haploid number). We expect that the methods will also be useful for future studies on the outcomes of human *in vitro* fertilization.

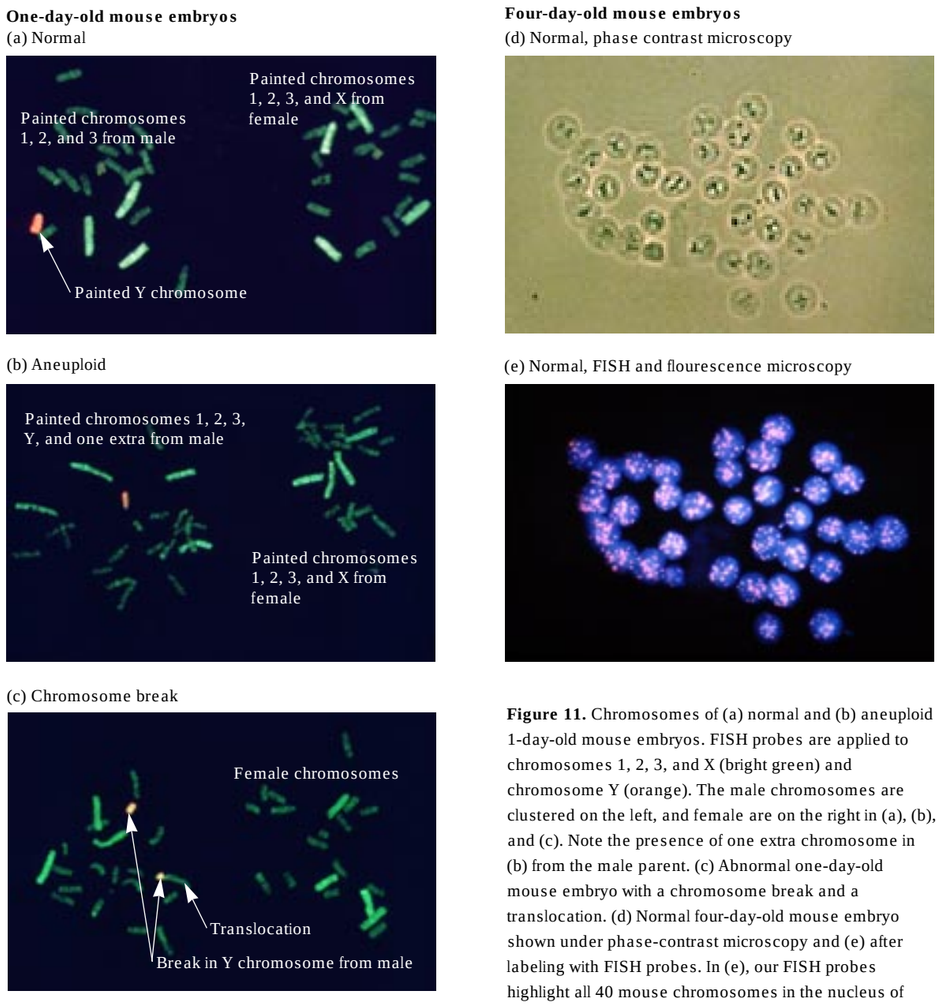


Figure 11. Chromosomes of (a) normal and (b) aneuploid 1-day-old mouse embryos. FISH probes are applied to chromosomes 1, 2, 3, and X (bright green) and chromosome Y (orange). The male chromosomes are clustered on the left, and female are on the right in (a), (b), and (c). Note the presence of one extra chromosome in (b) from the male parent. (c) Abnormal one-day-old mouse embryo with a chromosome break and a translocation. (d) Normal four-day-old mouse embryo shown under phase-contrast microscopy and (e) after labeling with FISH probes. In (e), our FISH probes highlight all 40 mouse chromosomes in the nucleus of each cell of the embryo.

New Research on Klinefelter Syndrome

We are beginning to look at blood samples from humans who carry the genetic abnormality associated with Klinefelter syndrome (47, XXY). Such individuals tend to be slower than normal in physical and behavioral development, they eventually grow taller on average, and they are all sterile. The work is a collaboration involving LLNL and five other institutions.

About half of 47, XXY cases receive the extra X chromosome from the father (such aneuploid sperm would carry both the X and Y chromosomes). Male children with this syndrome and their fathers provide us with a unique opportunity to learn about the relation between sperm aneuploidy and aneuploidy at birth. We will study 40 families with children whose diagnosis of Klinefelter syndrome has been genetically confirmed. We want to know if fathers who are responsible for the syndrome in their child produce inherently elevated levels of aneuploid sperm, especially XY sperm. To increase the speed of scoring defective chromosomes and the use of objective criteria in the FISH assay, we are also developing new automation and image-analysis techniques.

Looking Ahead

For decades, genetics researchers, concerned physicians, and many parents have struggled to come to terms with the causes and conditions that may underlie abnormal reproductive outcomes. With highly efficient FISH probes, we are beginning to understand some of the ways chromosomal abnormalities can arise in sperm and how those defects may lead to defects in the embryo.

Our expectation is that the new procedures will lead to a far greater understanding of the relations among certain genetic defects in human sperm, the effects of age, environmental exposures, and life-style factors, and the probability of fathering a chromosomally defective child. On the horizon are improved FISH assays for more chromosomes, new assays that can detect chromosome breakage in sperm, and automation and objective image processing. Such advances will help to make our methods more accessible to the rest of the research community.

Key Words: aneuploidy; chromosomal abnormality; DNA probes; fluorescence *in situ* hybridization (FISH); Klinefelter syndrome; sperm—human, rodent; sex chromosomes.

References

1. A. J. Wyrobek, *et al.*, "An Evaluation of Human Sperm as Indicators of Chemically Induced Alterations of Spermatogenic Function. A Report for the U.S. Environmental Protection Agency Gene-ToxProgram," *Mutation Research* **115**, 73 (1983).
2. U.S. Congress, Office of Technology Assessment, *Technologies for Detecting Heritable Mutations in Human Beings*, OTA-H-298, U.S. Government Printing Office, Washington, D.C. (1986).
3. E. Rudak, *et al.*, "Direct Analysis of the Chromosome Constitution of Human Spermatozoa," *Nature* **274**, 911 (1978).
4. Brigitte Brandriff, "Visualizing Chromosomes in Human Sperm," *Energy and Technology Review* (March 1984), UCRL-52000-84-3, pp. 1–12.
5. A. V. Carrano, "The Human Genome Project," *Energy and Technology Review* (April/May 1992), UCRL-52000-92-4/5, pp. 29–62.
6. W. A. Robbins, et al., "Three-Probe Fluorescence *In Situ* Hybridization to Assess Chromosome X, Y, and 8 Aneuploidy in Sperm of 14 Men from Two Healthy Groups: Evidence for a Paternal Age Effect on Sperm Aneuploidy," *Reproduction, Fertility, and Development* (in press).
7. X. Lowe, *et al.*, "Aneuploidies and Micronuclei in the Germ Cells of Male Mice of Advanced Age," *Mutation Research* (1995) (in press).

For further information contact Andrew J. Wyrobek (510) 422-6296 (wyrobek1@llnl.gov).

About the Scientist

ANDY WYROBEK joined the Biomedical Sciences Division of the Laboratory in 1975. He is currently the principal investigator of the sperm and embryo research team within the Biology and Biotechnology Research Program at the Laboratory. He received his B.S. in physics from the University of Notre Dame in 1970 and his Ph.D. in medical biophysics from the Ontario Cancer Institute at the University of Toronto in 1975. In more than 80 publications, Andy Wyrobek has explored male-mediated developmental toxicology, human male reproductive hazards, and mammalian testing systems for detecting the genetic effects of environmental, occupational, and therapeutic agents in sperm and embryos. His special interests are understanding the mechanisms leading to birth defects and identifying the environmental and genetic risk factors for abnormal pregnancies.