Chapter 6

In vitro validation of translocation frequency obtained by FISH with mFISH
6.1. Introduction

For the last 30 years, the chromosome aberration assay has been used for the estimation of genome damage caused by clastogens. Fluorescence in situ hybridization (FISH) has contributed immensely to this aspect. Until rather recently, it was usually assumed that virtually all chromosome exchanges are simple, i.e. involve only two chromosome breaks. However, chromosome “painting” techniques have now shown that complex aberrations, involving more than two breaks in a single configuration, are common. (1-4). Due to its speed and ease of use, many whole-chromosome painting techniques based on FISH have become a method of choice to visualize chromosomal aberrations. A colorful diversity of radiation-induced chromosome aberrations is now observed with chromosome painting techniques.

mFISH combinatorial painting is particularly informative (5-8) as all pairs of autosome homologues, as well as the sex chromosomes X and Y, are simultaneously assigned their own (pseudo-) color, so that a large majority of interchanges (aberrations involving two or more different chromosomes) become readily detectable (5-10). The intricate aberration spectra uncovered by mFISH give extra information about the mechanisms and geometric aspects of radiation damage.

Most quantitative aberration formation modeling has explicitly or implicitly assumed, that low LET radiation induces double-strand breaks (DSB) randomly and independently within the genome and that DSB misrejoining is random. These two randomness assumptions have long been debated, and remain controversial (11-12). One hypothesis claims that randomness of DSB misrejoining is modulated by ‘proximity’ effects as DSB interactions have a limited range (13). This influences observed aberration spectra (2, 14) because chromosomes are localized in territories (15-16).

In this regard, it was of interest to check whether radiation induced TL involve chromosomes in a random fashion or in a non random manner. Hence the data presented here concerns human peripheral blood lymphocytes cultured for 50 hours
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after 2Gy and 4Gy acute irradiation in vitro. Three different techniques were employed to study the TL spectra – G-banding, FISH and mFISH. Importance was given to translocations, mainly due to their stable nature and the fact that these cytogenetic techniques could easily identify TL.

Subsequently, resulting genomic frequencies have been compared and chromosomal participation in TL formation has been checked. The present study was conducted in order to bring out the power of mFISH, for the rapid identification of radiation-induced TL. Results from two other assays, namely the G-banding technique and FISH assay were compared to validate the utility of mFISH.

6.2. Methodology

Peripheral blood lymphocyte irradiation (2 Gy and 4 Gy, Co-60 gamma radiation), culture and slide preparations were done as described in detail in Sections 2.3.1.1., 2.3.1.3. mFISH was performed on metaphase spreads using commercially available WCP probes (Spectra Vysion Kit) following the manufacturers instructions (Section 2.3.1.6.). Hybridized metaphase spreads were analyzed using a computer-assisted fluorescence microscope Axioplan 2, Zeiss, Germany. Translocation frequencies were converted into genomic frequencies with the formula for correction established by Lucas et al (17) and further simplified by Braselmann et al (18). The FISH data and G-banding data for translocations at 2 Gy was taken from Tables 3.2 and 5.1 respectively.

Translocation yields at 2 Gy and 4 Gy radiation dose as ascertained by G-banding, FISH and mFISH were compared. The TL spectra at 2 Gy including involvement of chromosomes in aberration formation as delineated by G-banding and mFISH was also compared.

Statistical analysis like normalization, ‘t’ tests, etc was performed when necessary. All statistical analysis was done with the online Instat Graphpad software.
6.3. Results

Our mFISH analysis of the non-irradiated blood samples revealed neither structural nor numerical chromosome aberrations. After *in vitro* exposure to 2 Gy and 4 Gy, TL scored in metaphases by mFISH were tabulated and are given in Table 6.1. These TL yields were compared with those obtained by FISH and G-banding and have been depicted as a bar diagram (Figure 6.1). The yields obtained by each of the three techniques were analysed using the ‘t’ test. No significant difference was seen in the aberrations yields and obtained p values have been tabulated in Table 6.2.

Many of the individual aberrations in the pooled data were simple translocations, but many other aberrations were visibly more complex. Figure 6.2 is a representative of the highly complex nature of aberrations induced by high dose of low LET gamma radiation. The display of the five different fluorescent images revealed the chromosomal makeup of the aberrations, i.e., the origin of the different parts of the chromosome. We observed 2 translocations between 3 chromosomes – 8, 9 and 11. A piece of chromosome 2 was inserted into chromosome 13. Also, there was a dicentric involving chromosomes 3 and 14.

Further, chromosomal participation in TL has been depicted in Figure 6.3. The TL yields were normalized and then plotted as a function of chromosomal DNA content as given by Morton (19).
Table 6.1: Translocation yields obtained on scoring peripheral blood lymphocytes exposed to Co-60 gamma radiation as ascertained by mFISH

<table>
<thead>
<tr>
<th>Dose (Gy)</th>
<th>CS</th>
<th>GE</th>
<th>TL</th>
<th>TL / GE ± SE</th>
<th>TL Distribution</th>
<th>u</th>
<th>σ²/y</th>
</tr>
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<tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>0</td>
<td>110</td>
<td>104.94</td>
<td>0</td>
<td>0</td>
<td>110</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>50</td>
<td>47.70</td>
<td>8</td>
<td>0.1677 ± 0.0593</td>
<td>43, 6, 1</td>
<td>-</td>
<td>0.59, 1.114</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-</td>
<td></td>
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<tr>
<td>4</td>
<td>25</td>
<td>20.99</td>
<td>18</td>
<td>0.9484 ± 0.2176</td>
<td>8, 10, 2, 2, 0</td>
<td>1</td>
<td>-0.57, 0.822</td>
</tr>
</tbody>
</table>

CS – Cells scored        GE – Genomic Equivalents        TL – Translocation
SE – Standard Error       u – Dispersion Coefficient     σ²/y – relative variance

Figure 6.1: Translocation yields obtained by G-banding, FISH and mFISH in peripheral blood lymphocytes exposed to Co-60 gamma radiation.
Figure 6.2: Highly complex nature of aberrations induced by high dose of low LET gamma radiation (4 Gy) as delineated by mFISH

Table 6.2: p values (‘t’ test) obtained on comparison of translocation yields by: G-banding, FISH and mFISH

<table>
<thead>
<tr>
<th>Techniques compared</th>
<th>p value</th>
</tr>
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<tr>
<td>FISH / mFISH</td>
<td>0.3176</td>
</tr>
<tr>
<td>mFISH / G-banding</td>
<td>0.3680</td>
</tr>
<tr>
<td>FISH / G-banding</td>
<td>0.1994</td>
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</table>
Figure 6.3: Comparison of translocation involvement as obtained by mFISH and G-banding in peripheral blood lymphocytes exposed in vitro to different doses of 0.8Gy/min Co-60 gamma radiation as plotted against DNA content of each chromosome.

6.4. Discussion

mFISH (5) is a powerful tool to detect and identify radiation-induced chromosome aberrations (4, 6). Experimental data give direct information on all chromosomes and almost all interchanges in each metaphase, without extrapolation. Our results show that mFISH is suitable to rapidly and unambiguously identify radiation-induced chromosomal abnormalities of in vitro irradiated lymphocytes.

The major advantage of mFISH is the speed of the technique, which, is much faster than classical cytogenetic analysis using G-banding. Probes are commercially available, the hybridization proceeds overnight, and slide washes and image
acquisition are completed within 2 hours. Furthermore, cytogenetic expertise is very helpful but not necessary for the analysis of mFISH experiments.

mFISH also poses an advantage over FISH, because, currently FISH provides data only from a part of the genome, requiring analysis of thousands of metaphases to reduce the overall uncertainty for an individual assessment of doses, in particular at low-dose and protracted exposures. As with any technique, some disadvantages are that the technique needs to be optimized for the target material in order to obtain optimal hybridization quality, and that the results need to be double-checked.

Since complex chromosome aberrations are a major problem in scoring of FISH-painted chromosome-type exchange aberrations at higher doses, such findings are important at increasing doses and should also be considered if the temporal stability of cells carrying translocations is being investigated. These data also support the idea to use a whole genome analysis approach instead of partial genome analysis for applications in dose reconstruction. However, although complex aberrations might be misinterpreted in FISH-painting analysis the importance of such aberrations in the low dose range is negligible. Moreover, calibration curves and control frequencies do not exist for any of the whole genome analysis approaches so far. Although the cell numbers which are necessary for a statistically sound whole genome analysis are obviously lower than for a partial genome analysis such data cannot be established in a short time. The generation of whole genome data also requires special expertise to score, to analyse and to interpret the aberration patterns. Thus, whole genome approaches are not applicable for routine analysis. Moreover, the costs for whole genome analyses are much higher than for partial genome analyses due to the very expensive DNA probe mixtures and the need for special microscope and image analysis equipment. However, it seems reasonable to apply them if high dose exposures can be assumed to complete aberration data obtained from a partial genome analysis.

The results of in vitro irradiated lymphocytes Table 6.1, Figs. 6.1 and 6.2 show the immense yield of information resulting from mFISH experiments and the characterization of complex aberrations. The mFISH approach appears to be a
significant step towards a better understanding of radiation-induced chromosome changes. In another study, as many as 15 different chromosomes were found to be affected by exposure to 4 Gy of radiation (6). Such multiple damaged metaphases provide proof for the presence of the suggested ‘hidden complexity’, which can be expected after irradiation.

Inter-donor variability may explain some of the controversies regarding the inter-chromosomal distribution of radiation-induced aberrations (20). The interaction of a large number of chromosomes in exchange aberrations suggests that the chromatin in the territory of one chromosome is accessible for several other chromosomes and that this accessibility depends on the size of the chromosome. Chromosomes show different sensitivity to ionizing radiation. The difference is dependent on the aberration type and is not always reproducible. This variability could contribute to the difficulty in reaching a consensus regarding the radiosensitivity of individual chromosomes (21).

The results of the study carried out by Cafourkova et al. (22), demonstrate that the frequencies of exchange aberrations in individual chromosomes and also between other chromosomes are usually linearly proportional to the DNA content of chromosomes. Our results also support the random involvement of chromosomes in translocation formation proportional to the DNA content. These results are in accordance with a review combining many cytogenetic studies (both classical and molecular) to investigate whether radiation-induced misrejoining of chromosome breakpoints is randomly or non-randomly distributed through the human genome (12). The authors conclude that there is an approximately linear proportionality between autosomal DNA content and the observed breakpoint number after the low-LET irradiation. Most DSB induced by low LET radiation are not concentrated in the radiation track of a single particle and are homogeneously distributed throughout the genome. Hence, the homogeneity of DSB distribution in cells irradiated with low LET radiation would give rise to a uniform distribution of exchange aberration in the genome.
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The comparison of three different techniques of visualization and interpretation (G-banding, FISH and mFISH), to the best of our knowledge represents the first study carried out and also gives room to validate these techniques.

It recently has been proposed that the presence of cells containing clonal aberrations, may have a severe impact on study outcomes when using cytogenetic methods for radiation exposure assessments and that this justifies their careful evaluation. Our results suggest that mFISH enables one to investigate the clonality of chromosomal aberrations. Furthermore, many cryptic CA in cytogenetically normal appearing metaphases, can now be detected and the chromosomes involved can be identified. Nevertheless, it has to be noted that mFISH will not detect all TL: small insertions or translocations with a size below the detection limit of mFISH (5) or inversions might be missed.

In summary, we have demonstrated that the quantification of radiation-induced chromosome aberrations from the whole genome by mFISH revealed comparable results as when calculated from partial genome data by FISH-painting (with chromosomes 1 and 3) and classical G-banding analysis. It turned out that at high doses complex aberrations may be misinterpreted by FISH-painting as simple aberrations. Therefore, whole genome analysis of radiation-induced chromosome aberrations by mFISH is a helpful tool especially in the high-dose range (18).

6.5. Conclusion

The idea to apply mFISH for ‘biological dosimetry’ as it has been done previously with the two or three-color WCP FISH is quite intriguing. The application of mFISH is expected to result in a more detailed and informative picture of radiation effects. Eventually, this technique will allow researchers to rapidly delineate chromosomal breakpoints and facilitate the identification of the genes involved in radiation tumorigenesis.

In our study, in vitro validation of FISH based results with various cytogenetic techniques like the conventional G-banding and the modern era mFISH assay, gave
consistent results. As we were able to validate the *in vitro* usefulness of FISH, we were next concerned with *in vivo* validation of the same. Hence the next section deals with *in vivo* validation of TL yields obtained by FISH with G-banding.

### 6.6. References


