# Computer analysis of mFISH chromosome aberration data uncovers an excess of very complicated metaphases

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## ► Abstract

*Purpose.* To analyze spectra of chromosome aberrations induced *in vitro* by low LET radiation, in order to characterize radiation damage mechanisms quantitatively.

*Methods.* Multiplex fluorescence in situ hybridization (mFISH) allows the simultaneous identification of each homologous chromosome pair by its own color. mFISH data, specifying number distributions for color junctions in metaphases of human peripheral blood lymphocytes 72 hours after exposure *in vitro* to a 3 Gy γ-ray dose, were combined with similar, previously published results. Monte Carlo computer implementations of radiobiological models for chromosome aberration production guided quantitative analyses, which took into account distribution of cells among different metaphases and lethal effects or preferential elimination of some aberrations at cell division.

*Results and Conclusions.* Standard models of DNA damage induction/repair/misrepair explain the main trends of the data as regards the fraction of metaphases having a particular number of colors involved in color junctions. However, all standard models systematically under-predict the observed fraction of metaphases where a large number of different chromosomes participate in aberrations. An early appearance of chromosomal instability could explain most of the discrepancies.

## Introduction

*Chromosome Aberrations.* Cells exposed to ionizing radiation undergo DNA breakage and misrejoinings that lead to large-scale rearrangements of the genome, *i.e.* to chromosomal aberrations. Low LET irradiation during the G0/G1 phase of the cell cycle produces primarily chromosome (i.e. chromosome-type) aberrations, rather than chromatid-type aberrations. Chromosome aberration spectra help characterize DNA repair/misrepair pathways, and also overall chromatin architecture during cell-cycle interphase (Savage 1998). A colorful diversity of radiation-induced chromosome aberrations is now observed with chromosome painting techniques, revealing a higher degree of complexity than was previously assumed (Cornforth 1998, Sachs *et al.* 2000a, Savage 2000, Loucas and Cornforth 2001). Particularly informative is mFISH combinatorial painting (Speicher *et al.* 1996, Greulich *et al.* 2000, Cornforth 2001, Saracoglu *et al.* 2001) where, as in spectral karyotyping (SKY; Schröck *et al.* 1996), 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.

*Models***.** Biophysical models of chromosome aberration radiogenesis (review: Savage 1998) can help identify dominant molecular damage-processing pathways, give predictions about aberration spectra, and guide computer simulations of aberration time development. The

biophysical model for aberration formation during G0/G1 most commonly used in radiobiology is Breakage-and Reunion (BR, figure 1A). There is substantial evidence that BR plays a prominent role in aberration formation for mammalian cells and that the non-homologous end-joining underlying this model is a major pathway for processing radiation damage during G0/G1 (Cornforth 1990, Edwards 1997, Cornforth 1998, Kanaar *et al.* 1998, Takata *et al.* 1998, Richardson and Jasin 2000, Hoeijmakers 2001, Sachs *et al.* 2000c). Also frequently considered is the one-hit, Recombinational-Misrejoining (RM) model (review: Cucinotta *et al.* 2000) pictured in figure 1B; in this case the homologous repair pathway (Lieber 1998, Hoeijmakers 2001) is often considered to be the underlying mechanism. A third model often used is the Revell-type Exchange Theory (reviews: Savage 1998, Edwards 2002).

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*Randomness.* Most quantitative aberration formation modeling has explicitly or implicitly assumed, at least for low LET radiation and for averages over scales larger than 1 Mbp, the following two properties: 1) radiation induces double-strand breaks (DSBs) randomly and independently within the genome; 2) DSB misrejoining is random. These two randomness assumptions have long been debated, and remain controversial. Even apart from the proximity effects discussed in the next paragraph, and apart from effects that occur at scales < 1 Mbp, the two assumptions are not precisely accurate (Knehr *et al.* 1996). But they are good approximations (review: Johnson *et al.* 1998), adequate for present purposes. They are important tools in quantitative analyses, showing whether data indicate some specific deviation from randomness, and helping organize otherwise overwhelmingly intricate details (Savage and Papworth 1982, Sachs *et al.* 2000c).

*Proximity effects.* It is now known that randomness of DSB misrejoining is modulated by 'proximity' effects, *i.e.* by a bias for interactions of genomic loci which are close to each other within the interphase cell nucleus as compared to more distant loci (review: Savage 2000). Proximity effects occur because DSB interactions have a limited range (Kellerer and Rossi 1978). They influence observed aberration spectra (reviews: Sachs *et al.* 2000a, Ottolenghi *et al.* 2001) because chromosomes are localized in territories (reviews: Münkel *et al.* 1999, Cremer and Cremer 2001). Modifications of randomness due to proximity effects can be taken into account, approximately but systematically and quantitatively at least for scales > 1 Mbp, by using site or distance models of chromosome aberration formation (review: Sachs *et al.* 2000b).

*Monte Carlo computer analysis.* Modern aberration data sets are so detailed and complex that computer simulations (Lucas and Sachs 1993, Edwards *et al.* 1996, Cornforth 1998, Ottolenghi *et al.* 1999, Sachs *et al.* 2000a, Chatterjee *et al.* 2001) are important for their quantitative analysis. Computer simulation approaches, based on biophysical models and simplifying assumptions for aberration formation, complement classic approaches for elucidating repair/misrepair pathways (*e.g.* blocking pathways with drugs, using mutants deficient in specific pathways, or sequencing DNA). The simulations are quantitative, allowing estimates of which pathways *dominate* and which are minor.

We will use extensions of CAS (chromosome aberration simulator) Monte Carlo software, which implements standard aberration models assuming randomness modulated by proximity effects (Chen *et al.* 1997). For aberrations scored at the first metaphase after acute irradiation, CAS has allowed calculation of model predictions for a broad range of data in the literature, including

dose-response curves and detailed aberration spectra. It has quantified reasonable agreement of the BR model (figure 1A) with the data (Sachs *et al.* 2000c), but some discrepancies have been found, including an under-prediction of the frequency of the most complex aberrations (Sachs *et al.* 2000b).

*Selection effects.* The data presented here concern human peripheral blood lymphocytes cultured for 72 hours after acute irradiation *in vitro*. At this time many cells have already undergone one or more divisions post-irradiation (Hoffmann *et al.* 1999, Anderson *et al.* 2000, George *et al.* 2001, Pala *et al.* 2001). During cell division, some rearranged chromosomes can get lost and/or lead to cell death (Bauchinger *et al.* 1986, Savage 1995, Matsumoto *et al.* 1998, Pala *et al.* 2001). In fact, for intermediate and high doses, where multi-track action dominates, aberrations appear to be the main cause of clonogenic death in many cases (Davies and Evans 1966, Carrano 1973a, Bedford *et al.* 1978, Joshi *et al.* 1982, Cornforth and Bedford 1987, Franken *et al.* 1999), although for 1-track action, dominant at low doses, other causes of clonogenic death outweigh aberration formation (Sachs *et al.* 1997). Our modeling will systematically take into account loss or lethal effects during the first few cell divisions of particular aberrations, simple or complex.

*Chromosomal Instability.* Chromosomal instability is an important aspect of genomic instability, a complex phenomenon that is believed to be a driving force in carcinogenesis and tumor progression (Lengauer *et al.* 1998, Anderson *et al.* 2001, Gisselsson 2001). Chromosomal instability is characterized by marked numerical chromosome aberrations ( *e.g.* aneuploidy) and by gross structural rearrangements many cell divisions after the damage insult (Fodde *et al.* 2001, Jallepalli and Lengauer 2001).

Ionizing radiation can induce chromosomal instability (reviews: Morgan *et al.* 1996, Holmberg *et al.* 1998, Wright 1998, Limoli *et al.* 2001), although the mechanisms that govern it remain unknown. Most experiments have emphasized long-term effects persisting over many cell generations. However, early investigations gave evidence that extra damage may appear within a few generations after irradiation (*e.g.* for micronuclei: Molls *et al.* 1981, Molls and Streffer 1984). Recent mFISH studies (Cornforth 2001, Loucas and Cornforth 2001) revealed very complex chromosomal rearrangements occurring by the time of the first metaphase, which suggested the possibility of an early form of instability. The time-course of aberration formation found in recent premature chromosome condensation experiments is consistent with such early instability (Greinert *et al.* 2000, Sipi *et al.* 2000). We will consider the possibility that an early form of instability might account for a larger-than expected number of very complicated metaphases found in the present data, modeling a possible scenario where new chromosome-type aberrations can arise from unstable primary aberrations. Our computer simulations attempt to mimic how aberrations evolve in time, an approach important in the present context because time-progression, not just complexity, is the hallmark of chromosomal instability (Gisselsson 2001).

## ► Materials and Methods

*Experimental methods.* Published data (Greulich *et al.* 2000) were augmented using the previously established procedures. Peripheral blood samples were taken from 5 healthy human donors (3 males, 2 females, 32-35 years of age). The blood samples were exposed *in vitro* to an acute 3 Gy dose of 6 MeV photons (2.8 Gy/min, Siemens MX-2, Germany). Lymphocyte cultures were set up, and metaphases prepared 72 hours after radiation exposure. Metaphase spreads were prepared following a standard protocol (Rooney and Czepulkowski 1997). mFISH was applied to metaphases using the SpectraVysion™ system (Vysis, Downers Grove, USA; Applied Imaging, Santa Clara, USA). Scoring used fluorescence microscopy in combination with the appropriate filter sets and image analysis software. Ambiguities (Lee *et al.* 2001) were resolved using inverse-DAPI patterns. The number of aberrant chromosomes|colors (*i.e.* chromosomes as represented by their pseudo-colors, without distinctions between homologous chromosomes) was determined in each metaphase.

*1-color exchanges.* Some exchanges occur between homologue pairs, or within a single chromosome. Results include inversions within one chromosome, 1-color acentric and centric fragments, 1-color acentric and centric rings, homologue-homologue translocations and homologue-homologue dicentrics. Some of these 1-color exchanges are comparatively hard to detect experimentally. Moreover, exchanges within one chromosome are as yet less well understood theoretically than are interchanges (Sachs *et al.* 2000b). We therefore removed all 1-color exchanges from the data before the main computer analyses, and considered primarily color junctions, but checked that this removal did not influence our over-all conclusions.

*Combining data.* To emphasize results common to all (or at least most) human lymphocytes, and to increase the statistical power of the study, pooling of data was emphasized. For each donor, the new results were combined with the previous data. The five donor data sets were then tested against each other using the Kruskal-Wallis ordered test for combining into a single data set; Monte Carlo estimates for the exact (i.e. non-asymptotic) Kruskal-Wallis results were performed using StatXact (Cytel Software Corporation, Cambridge, MA, USA).

*Computer methods.* CAS Monte Carlo calculations, representing aberration time-evolution as a discrete-time Markov chain, have been summarized elsewhere (Sachs *et al.* 2000b). In brief, in the "site" versions of CAS used here, chromosomes are assigned at random to interaction sites in the cell nucleus; for low LET radiation, as here, reactive DSBs are assigned at random in the genome; simulated misrejoinings or restitutions occur at random, but only within one site. Simulated properties such as the total number of reactive DSBs in a cell nucleus, their specific location in the genome, the proximity of given pairs of chromosomes, etc., thus vary stochastically from cell to cell. Completeness is assumed, *i.e.* all simulated DSB free ends are eventually either restituted or misrejoined. This assumption is made because true incompleteness is rare at low LET (Savage 1976, Boei and Natajaran 1998, Wu *et al.* 1998, Fomina *et al.* 2000; compare however Pala *et al.* 2001).

CAS uses two adjustable parameters: the number $S$ of interaction sites per cell nucleus, and the average number $\delta$ of reactive DSBs per Gy. $S$ is determined primarily by the proximity effects discussed above, while $\delta$ is determined primarily by the cells' radiation sensitivity. Once the two parameters are adjusted for a particular cell type and radiation, CAS can specify which

chromosomes are involved in aberrations for each simulated cell, allowing direct comparisons with experimental data.

This study used site number $S=8$. It was also checked that using other values of $S$, from 8 to 15, representing the range previously found for other low-LET lymphocyte data sets (review: Sachs *et al.* 2000c), would not significantly alter the over-all conclusions. The other CAS parameter, $\delta$, was fixed by regarding in the published data set (Greulich *et al.* 2000) the fraction of metaphases showing 0 chromosomes|colors (after 1-color aberrations had been removed as discussed above) as a firm value. This $\delta$ value was then used after the data was properly pooled. Since $\delta$ is putatively dose-independent, $\delta$ values were compared with values previously obtained for other lymphocyte data sets at various low-LET doses.

*Modeling lymphocyte proliferation.* It was assumed that the cells observed at 72 hr post-irradiation were distributed among three post-irradiation metaphases $M$ ($M=1, 2,$ or $3$). In our main calculations (figures 2 and 4) we used a published metaphase distribution (Hoffmann *et al.* 1999) for lymphocytes subjected to 3 Gy of low-LET radiation, as follows: 30|50|20 for $M$ = $1|2|3$ respectively (*i.e.* 30% of the cells in the first metaphase, 50% in the second, 20% in the third). Importantly, robustness of the conclusions drawn using these values was checked by subsidiary calculations using the values 100|0|0, 0|100|0, 0|50|50, and several other cases intermediate between these three extreme cases. Robustness calculations ensure that other choices of the proliferation parameters do not alter the main conclusions of our studies.

*Modeling aberration transmission and cell lethality.* To take into account selection effects (Carrano 1973a, b, Bedford *et al.* 1978, Braselmann *et al.* 1986, Pala *et al.* 2001) for cells that have divided at least once post-irradiation (*i.e.* for $M>1$), we used extensions of the model of

Carrano and Heddle (1973) as implemented by Braselmann *et al.* (1986). The model involves 3 parameters, defined in terms of the behavior at the first post-irradiation cell division, and thus measured by comparing $M=2$ metaphases to those in $M=1$ (Bauchinger *et al.* 1986), as follows: A) the dicentric survival parameter $W$, where $1-W$ is the probability a simple dicentric will lead to death of both daughter cells; B) the acentric survival parameter $P$, with $1-P$ the probability specifying the direct lethal effect an acentric (*i.e.* a linear fragment with two telomeres and no centromere, or an acentric ring) has by itself; and C), the acentric transmissibility parameter $T$, with $2T$ specifying the probability that a cell with an acentric transmits one or two copies of the acentric to one or the other daughter cell (but not to both). More details on the model are in the Appendix. In most calculations we used the values $W=0.42$, $P=1.0$ and $T=0.41$ measured in (Bauchinger *et al.* 1986). Robustness of the conclusions was then analyzed by subsidiary calculations using various other values, again to check that our overall conclusions did not depend on the specific choice of parameters.

*Extending CAS and applying the extension.* CAS output was extended to include a cell-by-cell list of aberrations in mPAINT nomenclature (Cornforth 2001), and to give totals based on that list. The proliferation and transmission models described above were added as extra modules, using Monte Carlo algorithms. A new module was used to implement the contagious misrejoining (CM) pathway introduced below in the discussion section. Simulations involved at least 500,000 cells for each set of parameter values. Computed male/female differences were found to be very minor for this kind of output.

**Results**

*Data and data pooling.* Table 1 shows the new data, for 5 donors, that complements previously published data (Greulich *et al.* 2000). Table 2 shows the old and new data combined, with 1-color aberrations removed as described in the Materials and Methods section. The Kruskal-Wallis test for singly ordered Rx11 contingency tables, where R is the number of rows (corresponding to the number of donors) and 11 is the number of categories (0 colors, 2-10 colors, and > 10 colors), gave the following results. The hypothesis that the data in table 2 for the five donors are five samples from the same population is rejected, and similarly for the data for any combination of four donors that includes donor #2 ($p < 0.001$ in each case). The hypothesis that the data for the four donors excluding donor #2 are samples from the same population is not rejected ($p=0.30$). Inspection of table 2 indicates the qualitative reason for these results: the cells from donor 2 seem less radiosensitive (penultimate column, table 2). Our computer analysis therefore used pooled data from four donors. Data for donor #2 were regarded as outliers and not considered further, except to check that if all data had been pooled, the discrepancies discussed below would not disappear.

Many of the individual aberrations in the pooled data were simple translocations or dicentrics, but many other aberrations were visibly more complex. Unirradiated controls for table 2 consisted of 201 metaphases, of which 200 (*i.e.* 99.5%) had 0 chromosomes|colors involved, one had 2 chromosomes|colors involved, and none had 3 or more. The fact that many metaphases for irradiated cells involved moderately large numbers of aberrant chromosomes|colors and some involved a large number, while the maximum number in any one control metaphase was 2, led us

to quantify, trying to confirm or refute the suggestion that there is a higher frequency of very complicated metaphases than standard models predict.

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*Central calculation.* The quantitative analysis used one 'central' computer calculation for the four-donor pooled data and used various subsidiary calculations designed to test how robust the results of the central calculation were. For the central calculation we used the BR model (figure 1A) but, as discussed below, this choice was not crucial. The central calculation used the following values for the parameters described in Materials and Methods.

A) Proliferation/lethality parameter values determined in (Bauchinger *et al.*1986): dicentric cell survival parameter $W=0.42$; acentric transmission probability $T=0.41$; and acentric cell survival parameter $P=1.0$.

B) Percentages of observed metaphases belonging to generation number M=1|2|3 post-irradiation as *30|50|20*, respectively (Hoffmann *et al.* 1999).

C) In CAS: $S = 8$ interaction sites per cell nucleus based on earlier analyses (Sachs *et al.* 2000c); and $\delta = 2.47$ reactive DSBs per Gy based on the data of (Greulich *et al.* 2000).

Using these parameter values, chromosome|color distributions were calculated with CAS and compared with the data in table 2. The results are shown in figure 2 (BR columns). For easier visualization two graphs are presented: the first shows categories of metaphases with 6 chromosomes|colors or fewer involved in aberrations; the second shows the remaining, more complicated, cases. It is seen that the simulations do track the trend of the data, but that there are some discrepancies. The simulations (BR model) under-predict all categories with 6 or more

chromosomes|colors, and they over-predict the 3 chromosome|color category. The most complicated metaphase observed had 14 chromosomes|colors; for this case the observed frequency is (1/886) ~ $10^{-3}$ and CAS simulations predict a frequency of ~ $4\times10^{-5}$.

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*Other calculations.* Subsidiary calculations, varying models and/or parameters, were performed to study how general the discrepancies uncovered by the central calculation are. First, the RM model (figure 1B) was substituted for the BR model. All parameters except $\delta$ were kept the same, and we derived $\delta = 0.59$ reactive DSBs per Gy for the Recombinational-Misrejoining mechanism by the procedure described in Materials and Methods. It is seen in figure 2 (RM columns) that essentially the same discrepancies appear as before, and that for most categories the discrepancies are slightly worse.

Numerous additional CAS calculations, using other standard models and/or other parameter combinations as described in the Material and Methods section and the Appendix, showed that the same types of discrepancies persist (*e.g.* figure 3). It is thus confirmed that the main conclusions do not depend on the choice of parameters. In particular, the parameters fixed from the literature (cell proliferation, aberration transmission and lethality) do not affect the over-all conclusions, *i.e.* any other reasonable choice of these parameters leads to a good fit of the data except for the consistent under-prediction of very complicated metaphases.

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Auxiliary calculations also showed that $\delta = 2.47$, derived from the frequency of undamaged metaphases in the data of (Greulich *et al.* 2000), is also nearly optimal for fitting the relative frequencies of damaged metaphases in the combined data set.

**Discussion**

mFISH (Speicher *et al.* 1996) is a powerful tool to detect and identify radiation-induced chromosome aberrations (Greulich *et al.* 2000, Loucas and Cornforth 2001). For the first time experimental data give direct information on all chromosomes and almost all interchanges in each metaphase, without extrapolation. There is enough information that its computer analysis here gave quantitative tests for whether the aberration spectrum is consistent with current ideas on aberration formation.

*Standard model predictions.* Our central calculation gave the right order of magnitude for the most frequent categories and reproduced the general trend of the data (figure 2, BR columns) even though $\delta$ was obtained based only on the published data of Greulich *et al.* (2000), and all other parameters were taken from other data in the literature. This consistency suggests that the underlying assumptions, including the randomness assumptions, used in all versions of all the models are reasonable approximations. The calculated value $\delta = 2.47$ reactive DSBs per Gy is in approximate agreement with the values $\delta \sim 3$ reactive DSBs per Gy previously found for other low-LET experiments (review: Sachs *et al.* 2000c).

The central calculation did show a number of discrepancies with the data, especially an under-prediction of categories with more than 6 chromosomes|colors per metaphase. Numerous robustness checks (*e.g.* figures 2 and 3) showed that the discrepancies could not be removed by changing to other standard models or to other reasonable parameter values.

*Contagious misrejoining (CM) could increase complexity.* The fact that standard models under-predicted the categories with large numbers of chromosomes|colors (figures 2 and 3) is consistent with other recent quantitative results showing under-predictions of observed very complex aberrations for hard and soft x-rays (Sachs *et al.* 2000b, Sachs *et al.* 2000c), and with our preliminary calculations (not shown) for other FISH data (Fomina *et al.* 2000) and mFISH data (Loucas and Cornforth 2001).

What causes so many very complicated metaphases? The pattern of discrepancies in figure 2 led us to consider a mechanism in which some 'unstable' misrejoinings produce *additional* misrejoinings. Then the predicted histograms would be shifted partially to the right, ameliorating the discrepancies. One possibility is shown in figure 4, panel A. A first round of misrejoining, carried to completion, is followed by a *second* round of misrejoining involving putatively unstable first-round misrejoinings. Specifically, a (small) probability, treated as an adjustable parameter $\wp$, was assumed for each first-round misrejoining to 'attack' a random, otherwise undamaged location in the genome and induce a complete reciprocal exchange. Thus, intuitively speaking, some misrejoinings were modeled as contagious, and we call this a 'contagious misrejoining' mechanism (CM). Different first-round misrejoinings were assumed to act independently of each other during the second round. The second round was assumed to occur during G1 in the same cell cycle as the subsequently observed metaphase, producing

chromosome-type chromosomal aberrations directly, rather than chromatid aberrations as is often found for long term instability.

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To get a specific example, the attack probability $\wp$ was chosen as $\wp$=0.11, and $\delta$ was readjusted from 2.47 to 2.42 to fit the 0-color category, with the first round calculation otherwise exactly the same as the central calculation discussed in the results section. Results are shown as CM in figure 4, panel B. It is seen that the contagious-misrejoining scenario removes or ameliorates the under-predictions for all categories with 6 or more colors. However, note also that the CM fit for categories with less than 5 colors is somewhat worse than the BR fit. A corresponding calculation using RM for the first round of misrejoining instead of BR gave slightly better fits, (results not shown); however, in calculations on data more detailed than these, specifying frequencies of simple and of complex aberrations, BR has been found to be markedly superior to RM, as discussed in the Introduction above.

*Other possible scenarios.* While the quantitative fits are still not wholly satisfactory, the improvements shown in figure 4 suggest that a different, perhaps more elaborate kind of contagious misrejoining could perhaps produce the aberration spectra observed. It is easier to consider possible scenarios than to carry out satisfactory quantifications, as the following examples illustrate.

**A.** The calculations for the CM mechanism of figure 4 assumed that restitutions (*i.e.* rejoinings, with no alteration of the DNA sequence, or alterations only on scales <<1 Mbp) do not generate second-round misrejoinings. In effect, instability was attributed to large-scale

rearrangements, generated by interchanges between different chromosomes or by multi-Mbp intrachanges within one chromosome, as could occur if disruption of chromosome territories (Cremer and Cremer 2001) were one of the driving mechanisms for instability. However, there is some evidence that radiation-induced chromosomal instability observed many cell cycles post-irradiation may instead be a generalized effect for the whole genome, not localized to earlier gross misrejoinings (*e.g.* Trott *et al.* 1998). We therefore tried a model in which all rejoinings and misrejoinings, including restitutions, can be unstable. It was found, however, that then the *relative* proportions of simulated metaphases with different numbers of chromosomes|colors involved are not substantially changed, so that the typical discrepancies described above recurred once we readjusted the parameter $\delta$ appropriately.

**B.** By assuming attacks to be at random genomic locations, the CM pathway shown in figure 4 in effect assumes that between the first and second misrejoining rounds there has been enough time for major geometric rearrangements of the chromosomes (compare Greinert *et al.* 2000, Sipi *et al.* 2000, Cremer and Cremer 2001). Calculations using the alternate assumption, that at the time of the second round the same proximity associations which limited the first round are still present, failed to show the systematic increases, in average number of chromosomes|colors involved, which are needed to improve the fit to the data.

**C.** The finding of metaphases with up to 14 chromosomes|colors involved suggests the possibility of fewer, but more highly contagious instabilities. A few aberrations in a small number of cells would be ‘very’ unstable in that they would trigger several rounds of contagion; other aberrations within those cells and other cells would have normal behavior. Quantification of this scenario would have to involve additional adjustable parameters.

**D.** An interesting possibility of a quite different sort is the pre-formed association model recently discussed in (Savage 2000). In that model there are special regions in the interphase nucleus where parts of many different chromosomes are in close spatial proximity, and radiation-induced chromosome interchanges occur primarily at these pre-formed associations. This geometry could perhaps result in very complex aberrations and thus metaphases with many chromosomes|colors involved in aberrations. However, this model has not yet been quantified.

**E.** Another possibility to consider is differential cell cycle delays for heavily damaged *vs.* lightly damaged cells. It has been suggested that, even at low LET where each cell gets essentially the same number of DSBs initially, cells which develop many aberrations may suffer a larger cell cycle delay from radiation than do cells with few or no aberrations (Lloyd *et al.* 1977, Boei *et al.* 1996, Ritter *et al.* 1996, Boei *et al.* 1997, Anderson *et al.* 2000). This could mean that first-metaphase cells at 72 hours have a larger proportion of very complicated metaphases than would be expected on the basis of a model, such as the ones used above, which does not take differential delays explicitly into account. However, in the data that we are looking at, we do not have the first-metaphase cells in isolation. The question in this context becomes whether differential cell cycle delays would lead to the observed mix of cells having a higher proportion of complicated metaphases than modeled. The observed mix includes second and third metaphase cells (Lloyd *et al.* 1977, Boei *et al.* 1996, 1997, Anderson *et al.* 2000), and the only reason the observed mix would be enriched in very complicated cells is if such cells are very delayed first metaphase cells (cell division would eliminate most of them). One can thus calculate an upper bound to the enrichment of complicated metaphases by neglecting differential cell cycle delays but allowing all cells in the simulations to divide successfully, even the ones with many aberrations. Carrying out this calculation, we found that even in this extreme case, the

model predicts fewer very complicated metaphases than observed (compare Fig. 3). Thus it appears that differential cell-cycle delays cannot account for the excess of very complicated metaphases.

*Summary.* Computer simulations based on biophysical models and applied to mFISH data allowed a quantitative evaluation of DNA repair/misrepair pathways. This gave evidence for some mechanism that can generate extra chromosome aberration complexity comparatively soon after acute irradiation.  An early instability pathway is one possible explanation.

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## ► List of abbreviations

DSB, DNA double-strand break; mFISH, multiplex- (multifluor-, multicolor-) fluorescence in situ hybridization; CAS, chromosome aberration simulator; BR, Breakage-and Reunion (figure 1A); RM, Recombinational-Misrejoining (figure 1B); CM, Contagious-Misrejoining (figure 4 A).

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**Appendix: Modeling aberration transmission and cell lethality.**

The model of Carrano and Heddle (Carrano and Heddle 1973) for selection effects was generalized by Braselmann, Bauchinger and Schmid (Braselmann *et al.* 1986). In this context, for metaphase number $M=2$, values were found by the same group (Bauchinger *et al.* 1986) for the three adjustable parameters -- acentric survival ($P$), dicentric survival ($W$), and acentric transmission ($T$) probabilities -- as follows.

**A.** $P=1$, corresponding to a picture where an acentric (*i.e.* a linear fragment with two telomeres and no centromere, or an acentric ring) by itself has no significant lethal effect during the first post-irradiation cell division.

**B.** $W=0.42$, meaning that for a cell which contains one simple dicentric (and its accompanying acentric), and contains no other aberrations which affect survival, both daughter cells survive in ~42% of the cases (Bauchinger *et al.* 1986). The model, summarized in (Braselmann *et al.* 1986), assumes that then both daughters have the dicentric (but not necessarily the acentric) and that in the remaining 58% of the cases neither daughter cell survives.

**C.** $T=0.41$, meaning that the probability for a cell with an acentric to transmit one or two copies of the acentric to one or the other daughter cell (but not both) corresponds to 2T, and therefore is, as a percentage, ~82%.

Our central calculation used this parameter set and our subsidiary calculations used various others, including a 'full lethality' case ($T=0=P=W$, *i.e.* any deviation from 46 chromosomes,

with one centromere each, leads to death of both daughter cells at the first division), an 'unrestricted transmissibility' case ($P=1=W$, $T=0.5$), and several intermediate cases (compare figure 3).

For metaphases with more aberrations, and for metaphase number $M=3$, some extensions were used. It was assumed, as in previous models (Braselmann *et al.* 1986), that complete independence holds for different aberrations. This assumption can be implemented conveniently by using the standard polycentric weight $\pi$, defined as follows:

$\pi$ = dicentrics + 2×tricentrics + 3×tetracentrics +…+ centric rings + 2×(dicentric rings) + …

The survival probability for progeny of a cell with polycentric weight $\pi$, compared to progeny of a cell without any potentially lethal aberrations ($\pi = 0$) was taken as $W^{\pi(M-1)}$. For metaphase number $M=2$ (or $1$) this value agrees with the usual formula (Braselmann *et al.* 1986), and for $M=3$ it is a natural extension.

The probability that a cell retains at least one copy of an acentric for $M=3$ was taken as $T^2$. This value may underestimate the probability of acentric transmission. Presumably at the second post-irradiation cell division there are often 4 copies of an acentric, and using $T^2$ neglects the possibility that at least one copy could go to one daughter cell and at least one other copy could go to the other daughter cell. However, for our purposes, with 1-color aberrations being excluded from the data and only the presence or absence of particular color junctions rather than their number in a metaphase being considered, any such underestimate does not affect the analysis. Since $W<1$, progeny of a cell having a rearranged chromosome with more than one centromere are eventually eliminated on this model (*i.e.* for $M$ large), consistent with the fact that a dicentric

is a ‘non-transmissible' aberration. Nonetheless W=0.42 implies that the behavior during the first few cell divisions is different, and some dicentrics can survive for a while. A similar behavior is predicted in the case of acentric fragments (provided $P$ is slightly less than 1), but in this case clonal elimination is even slower.

**Table 1.** New data on chromosome|color distributions.

| Chromosomes\|colors per metaphase | Donor 1 male | Donor 2 male | Donor 3 female | Donor 4 female | Donor 5 male | Total metaphases |
|---|---|---|---|---|---|---|
| 0 | 23 | 44 | 25 | 31 | 28 | 151 |
| 1 | 9 | 7 | 11 | 18 | 8 | 53 |
| 2 | 25 | 21 | 27 | 22 | 25 | 120 |
| 3 | 18 | 15 | 18 | 10 | 13 | 74 |
| 4 | 15 | 9 | 23 | 13 | 8 | 68 |
| 5 | 11 | 2 | 10 | 11 | 8 | 42 |
| 6 | 2 | 1 | 8 | 5 | 8 | 24 |
| 7 | 5 | 2 | 1 | 5 | 2 | 15 |
| 8 | 3 | 0 | 1 | 1 | 2 | 7 |
| 9 | 2 | 0 | 0 | 0 | 0 | 2 |
| 10 | 1 | 0 | 1 | 0 | 0 | 2 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 |
| 12 | 1 | 0 | 0 | 0 | 0 | 1 |
| 13 | 0 | 0 | 1 | 0 | 0 | 1 |
| Aberrant metaphases | 92 | 57 | 101 | 85 | 74 | 409 |
| Total metaphases | 115 | 101 | 126 | 116 | 102 | 560 |

The number of metaphases in which a given number of chromosomes|colors are damaged is shown. For example, if a metaphase has a simple dicentric plus its accompanying acentric fragment both involving the same two non-homologous chromosomes, and the metaphase has no other visible damage, it contributes one to the category of 2 chromosomes|colors per metaphase (row "2"); but if the dicentric involves two homologues the contribution is to row "1" instead, since both homologues have the same

color. For uniformity with the table based on earlier data published in (Greulich *et al.*, 2000), 1-color aberrations were not removed from table I. However, when combining data to obtain table II, 1-color aberrations were removed from both data sets.

**Table 2. Distributions of aberrant chromosomes|colors (combined data)**

| colors per metaphase | d1 m ‡ | d3 f | d4 f | d5 m | total | mean % * | ± %* | SD %* |
|---|---|---|---|---|---|---|---|---|
| 0 | 70 | 81 | 88 | 73 | 312 | 35.2 | 1.60 | 2.8 |
| 2 | 73 | 68 | 77 | 71 | 289 | 32.6 | 1.58 | 1.9 |
| 3 | 12 | 15 | 6 | 15 | 48 | 5.4 | 0.76 | 2.0 |
| 4 | 30 | 34 | 32 | 20 | 116 | 13.1 | 1.13 | 2.3 |
| 5 | 10 | 11 | 10 | 14 | 45 | 5.1 | 0.74 | 1.1 |
| 6 | 10 | 13 | 5 | 9 | 37 | 4.18 | 0.67 | 1.5 |
| 7 | 6 | 4 | 5 | 3 | 18 | 2.03 | 0.47 | 0.6 |
| 8 | 5 | 0 | 3 | 1 | 9 | 1.02 | 0.34 | 1.0 |
| 9 | 3 | 0 | 2 | 1 | 6 | 0.68 | 0.28 | 0.6 |
| 10 | 0 | 1 | 1 | 0 | 2 | 0.23 | 0.16 | 0.3 |
| >10 | 2 | 1 | 0 | 1 | 4 | 0.45 | 0.23 | 0.4 |
| Aberrant† | 151 | 147 | 141 | 135 | 574 | 64.79 | 1.60 | 2.8 |
| Total | 221 | 228 | 229 | 208 | 886 | 100 | | |

| d2 m | mean % |
|---|---|
| 106 | 53 |
| 55 | 27.5 |
| 13 | 6.5 |
| 17 | 8.5 |
| 5 | 2.5 |
| 4 | 2 |
| 0 | 0 |
| 0 | 0 |
| 0 | 0 |
| 0 | 0 |
| 0 | 0 |
| 94 | 47 |
| 200 | 100 |

The number of metaphases in which a given number of chromosomes are damaged is shown. Chromosomes are represented by their (pseudo-)colors (i.e. not distinguishing between homologues; see table I for an example). All 1-color aberrations were removed from each category; in particular, the category of metaphases where only one color was involved in aberrations disappeared completely (refer to Materials and Methods). In the >10 row, there were 2 metaphases with 11 colors, one with 13, and one with 14.

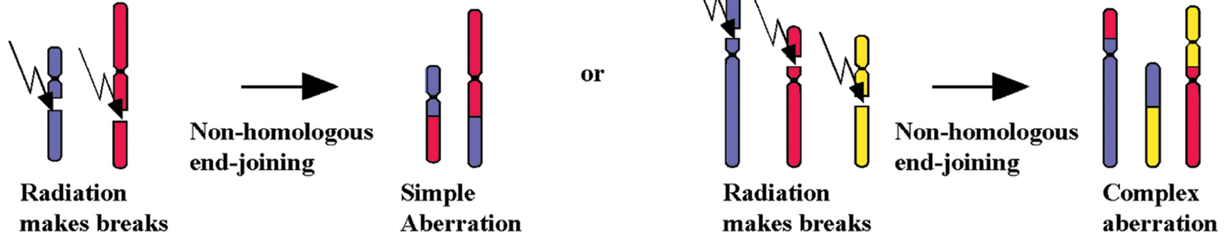
‡ “d1, m” stands for “donor 1, male”; “d3, f” stands for “donor 3, female”.

* The first column "mean" gives averages of 4 pooled donors (see materials and methods), represented as percentage of metaphases. For example, the total number of damaged metaphases involving exactly two (pseudo-)colors was 289 for all 4 donors, compared to 886 total metaphases; the corresponding percentage is $(100\times289/886) =$ 32.6%, shown as the mean for two colors. The column ± gives error bars for Figs. 2-4 based on binomial statistics. For example, for 2 colors per metaphase the ± entry is $100\times[p(1-p)/N]^{1/2}$ with N=886 and p=289/N. For comparison, the column SD is the sample standard deviation computed from the entries for the four donors with totals renormalized to 100; for example for 2 colors the entry is the standard deviation for $\{100\times73/221, 100\times68/228, 100\times77/229, 100\times71/208\}$.

† 2 colors or more.

The two columns on the right present the combined data, with 1-color aberrations removed, for the isolated donor (“d2, m”), and the corresponding mean values. Notice that only 47% of cells contained aberrations involving 2 or more colors, compared to ~65% for the other 4 donors.

**Figure 1.**



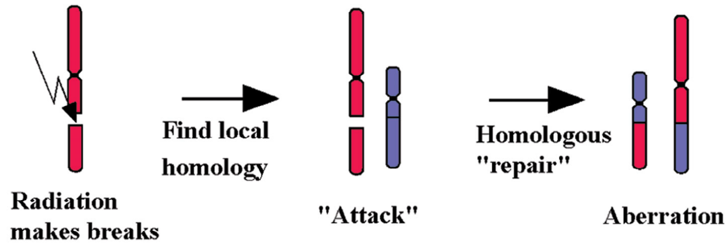
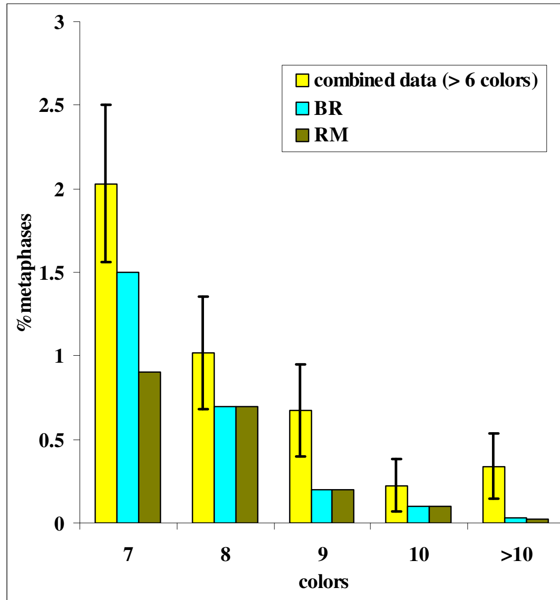



**Figure 2.**



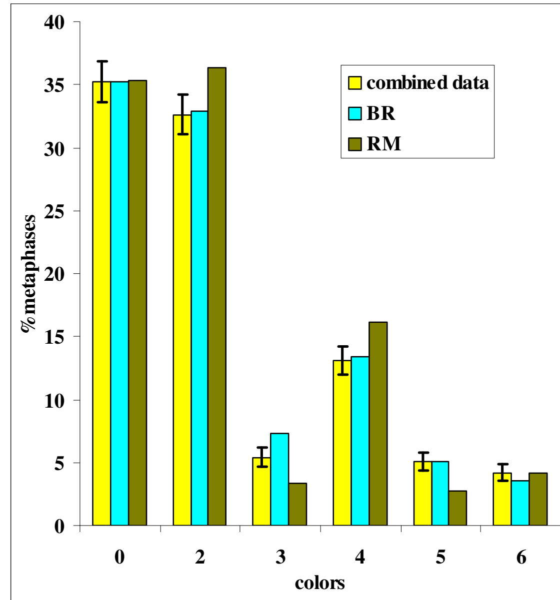
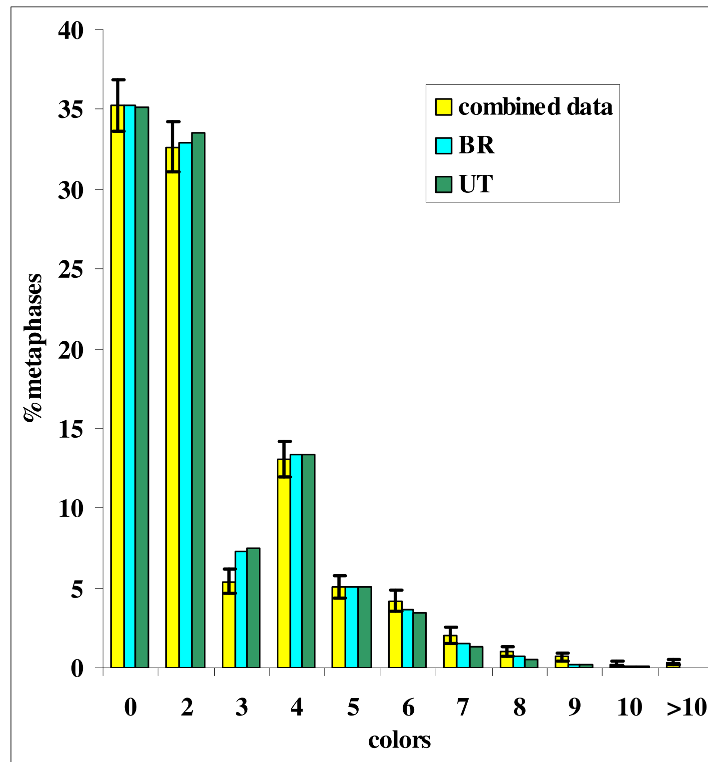



**Figure 3.**



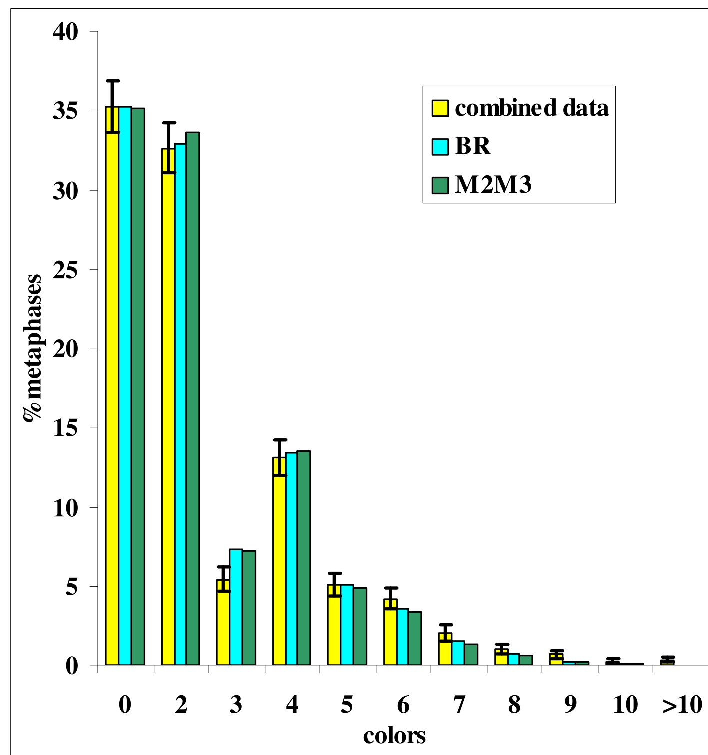



**Figure 4.**