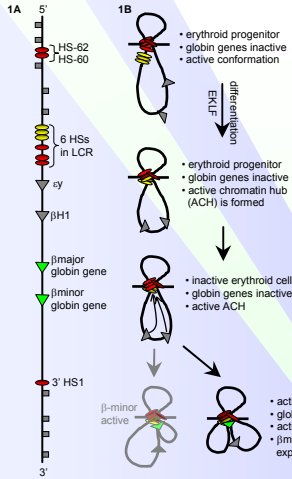


High-Resolution Confocal Microscopy and Nano-Scale Distance Measurements Show Difference in 3D Conformation Between Active and Inactive Mouse β -major Globin Loci?

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3C technology⁽¹⁾ shows that in erythroid cells the mouse β -major globin locus is folded in a looped structure during gene transcription. The looping is a result of clustering of DNase I hypersensitive sites (HS) from which intervening sequences loop out (fig. 1). This clustering of DNase I hypersensitive sites is however not present in non-erythroid cells.

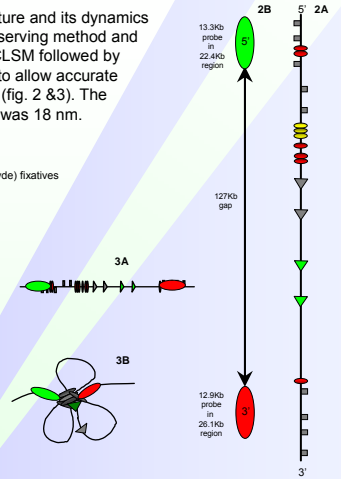
Fig 1: A: schematic map of the mouse β -globin locus. B: folding of the β -globin locus during various differentiation stages of erythroid cells. A distinctive 3D conformation is maintained during transcription of the globin genes in which the HSS, together with transcription factors form the core (or Active Chromatin Hub, ACH) of the structure.

To determine the actual 3D architecture and its dynamics we have established a structure preserving method and combined this with high-resolution CLSM followed by image restoration by deconvolution to allow accurate nano-scale distance measurements (fig. 2 & 3). The resolution equivalent of this method was 18 nm.

- 3D DNA-FISH protocol:**
- retaining chromatin structure using cross-link (aldehyde) fixatives
 - permeabilization treatments:
 - triton X-100
 - saponin
 - proteases
 - HCl
 - $N_2(l)$ freeze/thaw
 - hybridization with high quality labeled probes
 - post hybridization washes
 - embedding with correct anti-bleaching medium
 - finding the right balance for each application

Fig 2: A: schematic map of the β -globin locus B: location of the 2 probes used in 3D-DNA-FISH. The gap between the two probes is 127 Kb.

Fig 3: hypothetical difference of position of the 5' and 3' end probes when locus is either inactive ("linear", 3A) or active (folded, 3B).



Chromatic Shift

The extra-cellular chromatic shift (CS) was determined by scanning 50 500 nm multi-coloured TetraSpeck fluorescent beads (fig. 4A). The intra-nuclear CS was determined for each cell type by a 3D DNA-FISH using labeled telomere oligo probes (fig. 4B). The center of mass coordinates for each fluorescent signal was determined. The total CS (D) for beads and telomeres was calculated according to Pythagoras (fig. 5).

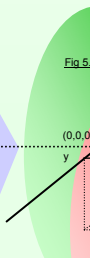


Fig 4: A: CS from 500nm beads (n=50):
 $D = \sqrt{((X_M - X_M)^2 + (Y_M - Y_M)^2 + (Z_M - Z_M)^2)}$
 $D_{beads} = 167 \pm 12$ nm
 of which the CS in x, y and z are:
 $X_{CS-beads} = X_M - X_M = 0 \pm 11$ nm
 $Y_{CS-beads} = Y_M - Y_M = -20 \pm 12$ nm
 $Z_{CS-beads} = Z_M - Z_M = -165 \pm 12$ nm

B: CS from telomeres in liver and brain cells:
 $D_{brain} = 210 \pm 60$ nm; $D_{liver} = 218 \pm 33$ nm
 of which the CS in x, y and z are:
 $X_{CS-brain} = -21 \pm 24$ nm; $X_{CS-liver} = -14 \pm 14$ nm
 $Y_{CS-brain} = 3 \pm 25$ nm; $Y_{CS-liver} = 1 \pm 16$ nm
 $Z_{CS-brain} = -206 \pm 61$ nm; $Z_{CS-liver} = 216 \pm 33$ nm

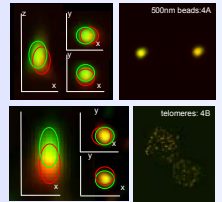
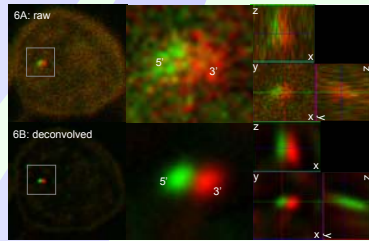


Image Restoration by Deconvolution

Confocal image stacks of fetal liver and control (brain) cells were deconvolved with the measured point spread function of the confocal microscope distilled from the average of at least 8 100nm green fluorescent beads or 200nm multi-coloured TetraSpeck beads.

Images were deconvolved using the classic maximum-likelihood estimation deconvolution algorithm that is implemented in the Huygens Professional image restoration, visualization and analysis software (SVI) (fig. 6). From each fluorescent signal the center of mass coordinates were determined and corrected for chromatic shift (fig. 7).



The Resolution Equivalent (RE) was determined. The RE for the 500nm TetraSpeck beads was 18 ± 8 nm. The RE for telomeres stained in fetal liver cells was 35 ± 17 nm and in brain cells 61 ± 34 nm.

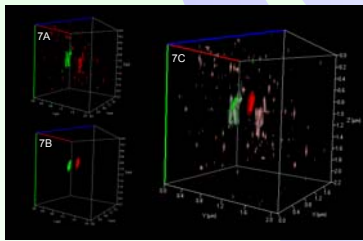


Fig 6 & 7: 3D DNA-FISH: Fig 6: A: one optical slice of a raw CLSM image stack showing fluorescent signals of one globin allele: 5' end stained green and 3' end stained red. Of the allele, a close-up as well as an xy, xz, and yz view are shown. B: same image slice after deconvolution of complete image stack. Fig 7: A: 3D projection of a raw image stack showing the 5' and 3' end of one globin allele in nuclear space. B: same 3D projection after deconvolution and chromatic shift correction. C: overlay of A & B.

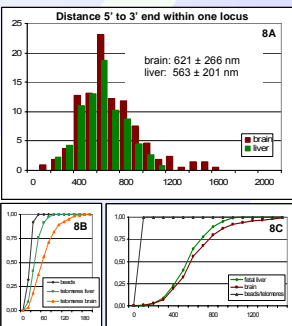


Fig 8: frequency (A) and cumulative frequency (C) distribution of distances between the 5' and 3' end of inactive (brain, red) and active (liver, green) β -globin locus. Control (beads and telomere distances (gray in B & C) cumulative distributions.

Results

Nano-scale distance measurements show that within an actively transcribing locus the distance between the 5' and 3' end of the locus is $563 \pm 201 \pm 12$ nm. In inactive loci the 5' to 3' distance is $621 \pm 266 \pm 16$ nm which is statistically significant. Since the spatial distance distributions are very similar we conclude that for both cell types the underlying 3D architecture is not dramatically different as earlier hypothesized⁽¹⁾ (see also fig. 3). However, frequency and cumulative frequency distributions (fig. 8A-C) show that the distance between the 5' and 3' end of the locus is more distinct and stable in erythroid (active) cells. In non-erythroid cells the distribution is broader, indicating a less distinct, more dynamic organized chromatin structure. These result agree with the hypothesis that gene activation requires a different/folded conformation of the chromatin than when genes are inactive. Now that only the nano-architecture seems to be functionally influenced by gene activation/transcription, this cannot be concluded on a larger scale. Inter-allelic differences do not show preference towards (interactive) chromatic co- and/or near-localization (fig. 9A & B).

Future

Distance measurements across this entire 218Kb chromatin region will elucidate the complete 3D structure of active and inactive mouse β -major globin loci. Furthermore, distance measurements combined with computer simulations (fig. 10) based on theoretical and/or empirical data, can give a better insight into high and low order chromatin architecture.

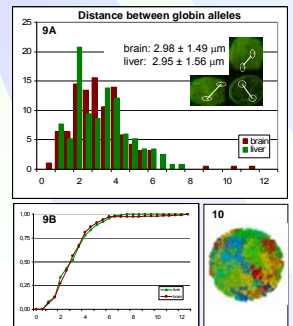


Fig 9: frequency (A) and cumulative frequency (B) distribution of distances between the two globin alleles in inactive (brain, red) and active (liver, green) cells. **Fig 10:** simulation of a full genome folded into a nuclear space that has a diameter of 10 μ m. Colours: different chromosome territories.

(1)Tobias et al. Mol Cell. 2002 Dec; 10 (6):1453-65 and Palstra et al. Nat Genet. 2003 Oct; 35 (2):190-195