Molecular combing and its application in clinical settings

Yiping Wang1,2, Kishore Ramesh Kumar1 and Thomas Liehr1*

Abstract
Molecular combing technology (MCT) is an effective means for stretching DNA molecules and making them thus accessible for in situ studies. MCT uses the force exerted in the process of liquid flow via surface tension to stretch DNA molecules and spread them on solid surfaces, i.e. glass cover slips. Many DNA molecules can be stretched at the same time in parallel and neatly arranged side-by-side, making the approach convenient for statistical analysis. Accordingly, DNA replication and transcription can be studied at the single molecule level. In this paper, the principle, experimental methods, important applications, advantages and shortcuts of MCT in medical field are presented and discussed.

Keywords: Molecular combing, Fluorescence in situ hybridization (FISH), Genomics, Constitutional genetic diseases, Acquired genetic diseases

Background
Molecular cytogenetics is the study of genomic alterations based on techniques associated with in situ hybridization. In the 1980's, fluorescence in situ hybridization (FISH) was developed from the radioactive variant of the technique and applied on human cytogenetic preparations [1]. At first, FISH seemed to be mainly useful to localize (human) genes; however, quickly the technology was adapted for clinical and tumor cytogenetics to characterize chromosomal rearrangements being unsolvable in banding cytogenetics (for review see [2]). At first, progress was driven by research-based laboratories, producing probes suited for FISH by cloning, glass-needle based chromosome microdissection or chromosome flow sorting [3]. These laboratories also introduced multicolor-FISH approaches like locus-specific probe based multiplex subtelomeric FISH [4], partial chromosome painting probe dependent multicolor banding (MCB) [5] or whole chromosome painting probe based spectral karyotyping (SKY) [6] and multicolor fluorescence in situ hybridization (M-FISH) [7]. Several of such probes and probe sets were also commercialized in parallel; for example MCB is available as mband-probe sets [8].

A limitation of chromosome-/metaphase oriented FISH is its power of resolution [2, 5]. Due to DNA compaction in metaphases it becomes difficult if not impossible to map the order of two or three genes along a chromosome if they are less than 2–5 Mb apart from each other [9]. In interphases DNA is more decondensed, still the order of three closely localized genes can only be determined reliably when evaluating 20–50 cells in a semi-statistical way; besides, the distance between them has to be in the range of 0.5 to 1 Mb or more. To achieve higher resolutions, approaches like fiber-FISH or molecular combing technique (MCT) were established [10–13]. In this review, MCT principle and how to perform, applications in medical field, advantages and shortcuts are presented and discussed.

*Correspondence: Thomas.Liehr@med.uni-jena.de
1 Institute of Human Genetics, Jena University Hospital, Friedrich Schiller University, Am Klinikum 1, 07747 Jena, Germany
Full list of author information is available at the end of the article

© The Author(s) 2022. Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article’s Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article’s Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit http://creativecommons.org/licenses/by/4.0/. The Creative Commons Public Domain Dedication waiver (http://creativecommons.org/publicdomain/zero/1.0/) applies to the data made available in this article, unless otherwise stated in a credit line to the data.
MCT—how it developed

The field of cytogenetics has focused in (i) medical genetics on studying the number, structure, function and origin of chromosomes and their abnormalities [2, 14], and (ii) in biology on the evolution of chromosomes [15]. The development of fluorescent molecules that either directly or via an intermediate-molecule bind to DNA [16] has led to the development of FISH, a technology linking cytogenetics to molecular genetics [2]. This technique has a wide range of applications that enlarged the possibilities of chromosome analysis [2]. The field of cytogenetics is particularly important for medical diagnostics and research as well as for gene mapping [2, 3]. Furthermore, the increased application of molecular biology techniques, such as array-based technologies, has led to improved resolution, extending the recognized range of microdeletion/microduplication syndromes and genomic disorders [17]. In adopting these newly expanded methods, cytogeneticists have used a range of technologies to study the association between visible chromosome rearrangements and defects at the single nucleotide level [18]. The development of molecular cytogenetic technology has increased the understanding of the possible molecular mechanisms involved in chromosomal rearrangements and genotype–phenotype associations, thereby helping patients to obtain better diagnosis and genetic counseling [2, 3].

FISH is a flexible technique that has driven the further development of different new molecular cytogenetic probe sets (see above) and/or applications. There are multiple approaches using FISH-based methods for different applications, like reverse-FISH [19], flow-FISH [20], Q-FISH (quantitative FISH) [21], cenM-FISH (centromere-specific M-FISH) [22], pod-FISH (parental origin determination FISH) [23], HCM-FISH (heterochromatin-oriented M-FISH) [24], and others. If modified, several FISH techniques can also be applied to interphase cells (interphase FISH) [25], which confers the advantages of FISH for the visualization of DNA probes in nuclei [26].

Different variants of FISH can be used to retrieve information on genomes from (almost) base pair to whole genomic level, as besides only second and third generation sequencing approaches can do [2]. Here especially to consider variations of FISH are chromosome orientation-FISH (CO-FISH) [27], Q-FISH [21], pod-FISH [22], FISH to resolve the nuclear architecture [9], multicolor-FISH approaches [2, 3], among other applied in chromoanagenesis studies [28] and MCT itself.

Fiber-FISH, also known as a MCT, hybridizes DNA probes to chromatin fibers stretched out on specimens, such as chromatin released from cells [10, 11]. An improved approach is to hybridize the probe with unfixed DNA fibers derived from cells embedded in pulsed-field gel electrophoresis clots. This method has been used for high-resolution gene mapping, gene replication, and direct observation of chromosomal breaks involved in translocations (see below for more details).

In 1994, Bensimon and coworkers [12] found that DNA could be uniformly straightened by a moving gas–liquid interface on a silanized substrate surface. They call this approach MCT, which can be used to straighten a large number of DNA molecules simultaneously and uniformly with a simple instrument (Fig. 1). As this procedure does not cause modifications in DNA sequence, it provides new possibilities to study the structure of DNA and especially the order of genes and loci. In the following substrate, straightening mechanism, pH condition, tension size were studied in detail to improve MCT [13].

As already implied before, MCT enables physical characterization of single genomes at the kilobase level of resolution over large genomic regions. An array of combed single DNA molecules is prepared by stretching molecules attached to a salinized glass surface with a receding air–water meniscus. By performing FISH on combed DNA, probe position can be directly visualized with respect to a closely located probe, enabling to

![Fig. 1 Scheme of molecular combing technique (MCT): 0.7 to 1 million of cells (either from cell culture or from peripheral blood lymphocytes) must be included in the experiment. Cells are collected and included in an agarose plug from which high molecular weight DNA is extracted. The latter can be applied for MCT itself and coverslips with DNA-fibers are produced. Coverslips with DNA-fibers (Fig. 2) can be used in standard FISH and obtained results can be evaluated using a fluorescence microscope](image-url)
construct physical maps and to detect micro-rearrangements (Fig. 1). Single-molecule DNA replication can also be monitored by detection of fluorophore labelled, incorporated nucleotide analogues on combed DNA molecules [29, 30]. Accordingly, problems to be solved in post genomic era can be faced thanks to MCT either via fluorescence (FM) and/or atomic force microscopy (AFM) [31].

MCT—principle

MCT takes advantage of physical or chemical binding forces between a DNA molecule and a hydrophobic surface. A solution with pure and high molecular weight DNA being arranged in an irregular coil shape, contacts the coverslip surface due to Brownian molecular movement. After attaching there, DNA is stretched by the retreating liquid surface, so that it is neatly arranged on the solid surface—here DNA changes its conformation from irregular coil to linear shape, driven by hydrophobic and/or electrostatic force [12].

MCT includes the following four steps: preparation of (i) Coverslips coated with a hydrophobic surfaces such as silane or polymethylmethacrylate and (ii) A high concentration DNA solution; the latter is prepared by embedding of the cells from which DNA is to be extracted in agarose plugs. After enzymatic treatment and washing, the pure and long DNA fibers as needed are prepared. (iii) Dipping and incubating the coated coverslip (from i) in the solution from (ii) for 5 min to bond the DNA to the coverslip. (iv) Pulling out the coverslip of the solution (from i) at a certain speed. This is a most critical step and must be done at steady speed of optimally 300 µm/s with a constant stretching factor (1 mm = 2 kb) [32–34]. Air drying fixes the DNA fibers to the surface.

The obtained coverslips are hybridized with certain FISH-probes (according to the question to be studied) and then evaluated at FM or AFM. This can be done either manually, or by a scanner, where the results can be evaluated statistically based on a special computer software (Genomic Vision, Bagneux, France) [32]. As the results obtained produce signal patterns of different lengths this kind of combination of “dashes and dots” is also referred to a „genomic Morse code “ (GMC) [35].

Advantages and restrictions of MCT

Clear advantages of MCT compared to other approaches is that it enables (a) visualization otherwise not accessible DNA-structures with (b) high sensitivity along single DNA-molecules of up to 12 Mb length [36]. (c) Regions from ~ 1 kb to 2 Mb can be studied applying FISH-probes which label 1 to 150 kb for deletions, duplications, amplifications and structural rearrangements, like inversions. (d) Results obtained are reliable and reproducible and MCT can accordingly be applied in clinical genetic diagnostics (see below). (e) As in other FISH based approaches multiplexing is possible, i.e. several loci can be accessed in parallel – only restriction are available fluorophores and number of filters in the detecting microscope (Fig. 2) or scanner [32].

Important limitations of MCT are, (A) that point mutations cannot be detected, (B) rearrangements below 1 kb in size may be missed, and (C) that commercial approach of MCT is limited to 2 to 3 probes, due to number of available detection filters in the commercially applied scanner [32].

Possible clinical applications of MCT

MCT has principally opened up new possibilities to detect submicroscopic, but by sequencing hard to access, complex DNA abnormalities. The latter can be related to inborn or acquired genetic diseases as well as viral infection, and thus MCT has several (potential) clinical applications already, which are summarized below.

MCT based clinical studies of gross chromosomal structures

While in plant genetics the possibilities to use MCT to characterize gross chromosomal structures (otherwise hard to access in detail) were already recognized early [33], in human such possibilities were only used occasionally. A literature review identified only three such studies: one being interested in the short arms of the

![Fig. 2](image-url)
acrocentric chromosomes and specifically the nucleolus organizing region [34], one characterizing a de novo microtriplication of 11q24.1 [35] and one to determine size of a microdeletion [36].

MCT based studies of familial adult myoclonic epilepsy 1 and 3
Familial adult myoclonic epilepsy 1 and 3 (FAME1 and FAME 3 – OMIM #601,068 and #613,608) are autosomal dominant inherited syndromes, being characterized by adult-onset cortical tremor, and may be associated with seizures. In Chinese and Japanese populations FAME1 has been found to be caused by enlarged intronic TTTTA/TTTCA repeats in SAMD12 gene in 8q24 [37, 38]. FAME3 is due to identical TTTTA/TTTCA repeat expansion in intron 1 of MARCH6 gene in 5p15.2 [39]. MCT has been proven to be able to detect and quantify these repeat amplifications [32].

MCT based diagnostics of facioscapulohumeral muscular dystrophy 1
Facioscapulohumeral muscular dystrophy type 1 (FSHD1- OMIM #158,900) is a disorder of skeletal muscles and shows (sometimes even within families) an extremely variable phenotype. In FSHD1, belonging to the group of hereditary progressive skeletal muscle dystrophies, a partial deletion of the D4Z4 repeats in 4q35 affects expression of DLX4 gene, as one copy of this gene can be found within each D4Z4 repeat [40]. Standard molecular diagnosis relying on Southern blot can be challenging because D4Z4 stretches are also present in 10q26. Nonetheless, by MCT D4Z4 comprising regions on chromosome 4 and 10 can be visualized separately; in contrast to other approaches MCT also enables clearly distinguishing of D4Z4 stretches on each individual chromosome 4 and 10 [41]. Thus, the CE (Conformité Européenne) certification for in-vitro diagnostics for an MCT based FSHD diagnostic assay was assigned to Genomic Vision, recently [32].

MCT based studies in cancer
In diagnostics of tumors, single-molecule methods can help to detect and study large DNA rearrangements that lead to cancer [42].

MCT based studies in leukemia

In a proof of principal study in 2016 Ittel and coworkers [43] showed, that MCT is well suited to identify variant breakpoints in “standard translocations” being associated with specific leukemia. Deviating breakpoint could be detected for translocation t(12;21)(p13;q22) involving ETV6 and RUNX1 genes, being typical for B-cell lineage childhood acute lymphoblastic leukemia.

MCT based studies of BRCA1 gene

A certain subset of hereditary breast and ovarian cancer is associated with germ line mutations of BRCA1 or BRCA2 gene. Accordingly, MCT has been used successfully for uncovering otherwise hard or not to detect combined small deletion / duplication events (in the range of 3 to 17 kb) in BRCA1 [44–46]. Also, ψBRCA1 pseudogene and a before unknown 100-kb sequencing gap upstream of the BRCA1 gene were identified by MCT. Even though more research studies with MCT concerning BRCA1 gene were undertaken in between [47], a standard application in tumor genetic diagnostics was not established yet.

MCT based studies of telomere length
Telomeres are specialized nucleoprotein structures at the ends of the linear chromosomes that function to protect the chromosome ends, thereby maintaining the stability of the genome. Telomeric DNA comprises repetitive sequences of the hexanucleotide TTAGGGa repeat unit, bound in a sequence-specific manner to the protein complex shelterin, and assembled into macromolecular structures called telomere-loops (t-loops). In normal human somatic cells, telomeres range from 5–15 kb in length, and length variability was found for individual telomeres and different cell types. Inter-individual variability is also observed across the human population, superimposed to the well-established age-associated decline in telomere length [48]. Possibilities and advantages of MCT to check telomere length are summarized by Kahl et al. [49].

MCT based diagnostics of viral integration
In terms of viral infection, the detection of foreign, viral DNA and its integration mode is intuitive and accurately possible by MCT. Especially, human papillomaviruses (HPVs) are frequently integrated in cancers. HPV genomes having a size of 7 to 8 kb, can be integrated as (type I) a single HPV genome, (type II) multiple, tandemly integrated HPV genomes, and (type III) multiple, tandemly integrated HPV genomes interspersed within host DNA [50]. Several MCT based studies for HPV integration [51–53] came to the conclusion that patients could benefit from this was of analyses, as subgroups based on viral integration sites could be established [53].

MCT based studies of population specific polymorphisms
MCT can even be used to gain insights into population specific differences, yet suggested to be mainly polymorphic variations, which may in future be attributed
to be associated also with susceptibilities to certain diseases. One study was on human blood neutrophil peptides (HNP1-3) and how copy number variants of alpha-defensins genes DEF A1 and DEF A3 vary and if they may be associated with infections and auto immune disorders [54]. In a second study analyzing CNVs of the human amylase gene clusters, MCT revealed unexpected genomic rearrangements leading finally to genomic instability, amplification and relocation of AMY2A and AMY2B genes. Here an association with obesity is suggested [55].

**MCT based research of DNA-replication**

All afore mentioned applications are based on the GMC-type evaluation. Besides, MCT also enables combining GMC with a replication combing assay (RCA). Thus, DNA synthesis kinetics of a specific replicating sequence can be compared with the remainder replicating genome. Yet, there are many research studies in model systems like *Saccharomyces*, *Xenopus*, or human cancer cell lines published, accessing replication kinetics of mitochondrial DNA, fragile sites or telomeres (for review see [32])); however, no applications in clinical setting are available yet, and thus not topic of this review.

**Conclusions**

MCT has great potential as an important cytogenomic tool in the field of chromosomic diagnostic and research [2]. Research applications of MCT mainly depend on research funds, which may be acquired more or less easily, if the underlying idea and project are of good quality. Introduction of MCT in diagnostics need to be at first approved by local authorities, like achieved for FSHD-diagnostics already; still the second big bottleneck is to find a way to get new methods into the national reimbursement catalogues. But, as MCT enable yet not, or by other means more complicated and more expensive cytogenomic approaches, there is to be expected a positive development.

**Abbreviations**

AFM: Atomic force microscopy; CE: Conformitè Européenne; cenM-FISH: Centromere-specific M-FISH; CO-FISH: Chromosome orientation-FISH; FAME: Fluorescence microscopy; FISH: Fluorescence in situ hybridization; FM: Fluorescence microscopy; GMC: Genomic Morse code; HCM-FISH: Heterochromatin-oriented M-FISH; MCT: Molecular combing technology; MCB: Multicolor banding; M-FISH: Multicolor-FISH; pod-FISH: Parental origin determination FISH; Q-FISH: Quantitative FISH; RCA: Replication combing assay; SKY: Spectral karyotyping.

**Acknowledgements**

Yiping Wang want to thank for the guidance and support of Thomas Liehr in developing the paper and his team for support. In addition, she is indebted to Jinze Group Beijing Jinze Medical Laboratory members.

**Author contributions**

YP had the idea for this review and drafted the paper; TL and KRK provided additional literature review data and revised the paper. KRK provided Fig. 2. TL finalized the draft. All authors approved the final version of the paper.

**Funding**

Open Access funding enabled and organized by Projekt DEAL. Furthermore, Yiping Wang holds a PhD fellowship of the Beijing Jinze Medical Science and Technology Development Co., Ltd, China.

**Availability of data and materials**

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

1 Institute of Human Genetics, Jena University Hospital, Friedrich Schiller University, Am Klinikum 1, 07747 Jena, Germany. 2 Jinze Group Beijing Jinze Medical Laboratory, Building A, National Engineering Center for Protein Drugs, Building 1, 33 Science Park Road, Huilongguan Town, Changping District, Beijing 1F, China.

Received: 18 October 2022   Accepted: 25 October 2022

**Funding**

Open Access funding enabled and organized by Projekt DEAL. Furthermore, Yiping Wang holds a PhD fellowship of the Beijing Jinze Medical Science and Technology Development Co., Ltd, China.

**Availability of data and materials**

Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

**Declarations**

**Ethics approval and consent to participate**

Not applicable.

**Consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests.

**Author details**

1 Institute of Human Genetics, Jena University Hospital, Friedrich Schiller University, Am Klinikum 1, 07747 Jena, Germany. 2 Jinze Group Beijing Jinze Medical Laboratory, Building A, National Engineering Center for Protein Drugs, Building 1, 33 Science Park Road, Huilongguan Town, Changping District, Beijing 1F, China.

Received: 18 October 2022   Accepted: 25 October 2022

**Published online: 16 November 2022**

**References**

1. Pinkel D, Gray JW, Trask B, van den Engh G, Fuscoe J, van Dekken H. Cytogenetic analysis by in situ hybridization with fluorescently labeled nucleic acid probes. Cold Spring Harb Symp Quant Biol. 1986;51( Pt 1):151–7.
2. Liehr T. Molecular cytogenetics in the era of chromosomics and cytogenomic approaches. Front Genet. 2021;12: 720507.
3. Liehr T. 2022. Basics and literature on multicolor fluorescence in situ hybridization application. http://cs-it.de/DB/TC/mFISH/0-Start.html (accessed on 11. October 2022)
4. Brown J, Horsley SW, Jung C, Saracoglu K, Janssen B, Brough M, Daschner M, Beedgen B, Kerkhoffs G, Eils R, Harris PC, Jauch A, Kearney L. Identification of a subtle t(16;19)(p13.3;p13.3) in an infant with multiple congenital abnormalities using a 12-colour multiplex FISH telomere assay M-TEL. Eur J Hum Genet. 2008;9:903–10.
5. Liehr T, Heller A, Starke H, Rubtsov N, Trifonov V, Marsek K, Weise A, Kuechler A. Microdissection based high resolution multicolor banding for all 24 human chromosomes. Int J Mol Med. 2002;9:335–9.
6. Schröck E, du Manoir S, Veldman T, Schoell B, Wenberg J, Ferguson-Smith MA, Ning Y, Ledbetter DH, Bar-Am I, Soenksen D, Garini Y, Ried T. Multicolor spectral karyotyping of human chromosomes. Science. 1996;273:494–7.
7. Speicher MR, Gwyn Ballard S, Ward DC. Karyotyping human chromosomes by combinatorial multi-fluor FISH. Nat Genet. 1996;12:368–75.
8. Castillo Taucher S, Fuentes AM, Paulos A, Pardo A. Multiple FISH y múltiple BAND: técnicas de citogenética molecular en cinco casos [Multiple FISH and multiple BAND: application of cytogenetic and molecular techniques in 5 cases]. Rev Med Chil. 2002;130:511–8.
9. Weise A, Starke H, Heller A, Claussen U, Liehr T. Evidence for interphase DNA decondensation transverse to the chromosome axis: a multicolor banding analysis. Int J Mol Med. 2002;9:359–61.
47. Tessereau C, Busson M, Monnet N, Imbert M, Barjhoux L, Schluth-Bolard C, Sanlaville D, Conseiller E, Ceppi M, Sinilnikova OM, Mazoyer S. Direct visualization of the highly polymorphic RNU2 locus in proximity to the BRCA1 gene. PLoS ONE. 2013;8: e76054.

48. Gomez DE, Armando RG, Farina HG, Menza PL, Cerrudo CS, Ghiringhelli PD, Alonso DF. Telomere structure and telomerase in health and disease (review). Int J Oncol. 2012;41:1561–9.

49. Kahl VFS, Allen JAM, Nelson CB, Sobinoff AP, Lee M, Kilo T, Vasireddy RS, Pickett HA. Telomere length measurement by molecular combing. Front Cell Dev Biol. 2020;8:493.

50. McBride AA, Warburton A. The role of integration in oncogenic progression of HPV-associated cancers. PLoS Pathog. 2017;13: e1006211.

51. Redmond CJ, Fu H, Aladjem MI, McBride AA. Human Papillomavirus Integration: Analysis by Molecular Combing and Fiber-FISH. Curr Protoc Microbiol. 2018;51: e61.

52. Warburton A, Redmond CJ, Dooley KE, Fu H, Gillison ML, Akagi K, Symer DE, Aladjem MI, McBride AA. HPV integration hijacks and multimerizes a cellular enhancer to generate a viral-cellular super-enhancer that drives high viral oncogene expression. PLoS Genet. 2018;14: e1007179.

53. Bouchilloux S, Fer F, Lemée F, Baradreau S, Dvorák V, Kubickova S, Ventru P, Tachezy R, Trnková M, Janda P, Abscheidt J, Amnial E, El Mhali D, Garcia F, Waisz M, Pilger G, Bensimon A, Mahé F. Correlation between integration of high-risk HPV genome into human DNA detected by molecular combing and the severity of cervical lesions: first results of the EXPL-HPV-002 study. Ceska Gynekol. 2019;84:84–92.

54. Hughes T, Hansson L, Akkouh I, Hajdarevic R, Bringsli JS, Torsvik A, Inderhaug E, Steen VM, Djurovic S. Runaway multi-allelic copy number variation at the α-defensin locus in African and Asian populations. Sci Rep. 2020;10:9101.

55. Shwan NAA, Louzada S, Yang F, Armour JAL. Recurrent Rearrangements of Human Amylase Genes Create Multiple Independent CNV Series. Hum Mutat. 2017;38:532–9.

56. Heiskanen M, Kallioniemi O, Palotie A. Fiber-FISH: experiences and a refined protocol. Genet Anal. 1996;12:179–84.

Publisher’s Note
Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.

Ready to submit your research? Choose BMC and benefit from:
• fast, convenient online submission
• thorough peer review by experienced researchers in your field
• rapid publication on acceptance
• support for research data, including large and complex data types
• gold Open Access which fosters wider collaboration and increased citations
• maximum visibility for your research: over 100M website views per year

At BMC, research is always in progress.
Learn more biomedcentral.com/submissions