Title
Quantitative DNA Fiber Mapping in Genome Research and Construction of Physical Maps

Author
Chu, Lisa W.

Publication Date
2005

Quantitative DNA Fiber Mapping in Genome Research and Construction of Physical Maps

Heinz-Ulrich G. Weier and Lisa W. Chu

Department of Genome Science, Life Sciences Division, University of California,

E. O. Lawrence Berkeley National Laboratory, Berkeley, CA, USA.

Correspondence to:

H.-U. Weier, Ph.D.

Life Sciences Division, MS 74-157

University of California, E.O. Lawrence Berkeley National Laboratory

1 Cyclotron Road, Berkeley, CA 94720, USA.

Phone: (510) 486 5347, fax: (510) 486 6746, e-mail: ugweier@lbl.gov

Running title: DNA Fiber Mapping

Prepared for: Methods in Molecular Biology, Humana Press Inc.

Abstract

Efforts to prepare a first draft of the human DNA genomic sequence forced multidisciplinary teams of researchers to face unique challenges. At the same time, these unprecedented obstacles stimulated the development of many highly innovative approaches to biomedical problem solving, robotics and bioinformatics. High resolution physical maps are required for ordering individual segments of information for the construction of a comprehensive map of the entire genome. This article describes a novel way to identify, delineate and characterize selected, often small DNA sequences along a larger piece of the human genome. The technology is based on immobilization of high molecular weight DNA molecules on a solid substrate (such as a glass slide) followed by uniform stretching of the DNA molecule by the force of a receeding meniscus. The hydrodynamic force stretches the DNA molecules homogeneously to approximately 2.3 kb/μm, so that distances measured after probe binding in μm can be converted directly into kb distances. Out of a large number of applications, this article focusses on mapping of genomic sequences relative to one another, the assembly of physical maps with near kilobase (kb) resolution and finally, quality control during physical map assembly and sequencing.

Key Words: Genome research; physical map assembly; DNA molecules; DNA fibers; fluorescence in situ hybridization (FISH); multi-color analysis; digital image analysis.

1. Introduction

High resolution physical maps are indispensable for large-scale, cost-effective DNA sequencing and disease gene discovery. Thus, the construction of high resolution physical maps of the human genome was one of the major goals of the human genome project in an effort to assemble a first draft of the human genome sequence (1). Prevalent physical mapping strategies for most organisms studied in recent years implemented a bottom-up approach for organizing individual pieces of DNA sequence information (typically provided in the form of recombinant DNA clones from large genomic libraries) into a high-resolution map of contiguous overlapping fragments (contigs). Large-scale sequencing, under way for various organisms, will greatly benefit from further progress in the creation of contig maps in a form suitable for DNA sequencing (2,3). A particular challenge to physical map assembly are regions rich in DNA repeats such as the centromeres of various vertebrates including man (4).

The cost-efficient sequencing of complex genomes also requires innovative approaches towards reducing the redundancy of sequencing templates which can further reduce the cost of the overall project. Template redundancy is caused by extensive overlap between sequencing templates (clones) and the presence of cloning vector sequence in these clones. This redundancy can be minimized by construction of physical maps of highest possible resolution and depth, definition of tiling paths comprised of minimally overlapping clones and knowledge of the extent of overlap between them, and characterization of sequencing templates prior to sequencing to reject clones that contain only cloning vector sequence or that are truly chimeric.

The recent progress in cloning large, megabasepair (Mbp) size genomic DNA fragments in yeast artificial chromosomes (YACs; 5-7) made it possible to rapidly construct low resolution framework maps based on overlapping YAC clones. Fluorescence in situ hybridization (FISH) has proved indispensable for identification of non-chimeric YAC clones and for physical mapping of individual YAC clones onto metaphase chromosomes (8). To generate physical maps with a resolution of 400 kb or better, the YAC clones were ordered by combining different complementing analytic techniques including pulsed field gel electrophoresis (PFGE; 9), FISH with interphase cell nuclei or metaphase spreads (10-13), STS content mapping (5,14-17), optical mapping (18,19) and/or DNA repeat fingerprinting (20-23). These approaches have been successfully used to assemble YAC contigs covering most of the human genome (24,25).

High resolution maps providing ordered sets of cloned DNA fragments at the 100 kb level of resolution are assembled with smaller, more manageable DNA fragments isolated from other libraries. Most mapping and sequencing groups prefer cloning of the genomic DNA in vectors which maintain relatively large DNA fragments without rearrangements, that are non-chimeric and allow easy DNA purification. In general, high resolution maps are comprised of overlapping cosmids (26-30), P1/PAC clones (31,32) or bacterial artificial chromosomes (BACs; 33).

Assembly of high resolution maps requires identification of cloned DNA sequences that contain overlapping regions of the genome. To minimize the effort invested in contiging a clone set, overlaps need to be determined quickly and accurately. This has been accomplished by various forms of clone fingerprinting (i.e., by identification of

common restriction fragment or inter-Alu PCR patterns (29,34-38)), by hybridization clone arrays bound to filters (21,39,40) or oligonucleotide arrays (41) and by identification of overlapping sequence tagged sites (STS; 42-44). The development of radiation hybrid (RH) maps (45) and the availability of large numbers of STS markers, together with extensive bacterial clone resources, provide additional means to accelerate the process of mapping a chromosome and preparing clone contigs ready for sequencing (Fig. 1; 46). These techniques, while effectively used by the genome community, are limited because they do not readily yield information about contig orientation, extent of deletions or rearrangements in clones, overlap of contig elements or their chimerism status nor do they provide information about the extent of gaps in the maps.

At LBNL, physical maps have been constructed for specific regions of the human genome. The typical procedure for physical map assembly at LBNL involved construction of a low resolution framework map based on YAC clones selected from the CEPH YAC library (6) using publicly accessible STS content mapping and fingerprint data (Fig. 1A; 3). High resolution physical maps were then built with P1/PAC or BAC clones. The clones were isolated initially by screening filters containing library copies with inter-Alu PCR products prepared from non-chimeric YAC clones (4), and later by PCR-based library screening (3). Next, more STS's were generated by clone endsequencing and used to screen the libraries for additional clones. Finally, physical maps were assembled based on clone overlap as indicated by STS content (Fig. 1A).

Techniques to rapidly identify minimally overlapping clones and to determine the extent as well as orientation of overlap will expedite the construction of minimal tiling

paths, facilitate the sequence assembly process, and lower the overall cost of the project. FISH can provide important information for high resolution physical map assembly. For example, FISH to interphase nuclei allows probes to be ordered with several 100 kb resolution (10-12), and FISH to preparations of decondensed nuclear (47,48) or isolated cloned DNA (49) allows visualization of probe overlap and provides some information about the existence and size of gaps in the map. However, none of these techniques provides quantitative information about the extent of clone overlap or about the separation between map elements because the chromatin on to which clones are mapped is condensed to varying degrees from site to site in these preparations.

The work of A. Bensimon and colleagues (50) showed that the extent of DNA condensation can be controlled by using a process termed 'molecular combing'. In molecular combing, a solution of purified DNA molecules is placed on a flat surface prepared so that the DNA molecules slowly attach at one or both ends. The DNA solution is then spread over a larger area by placing a coverslip on top. DNA molecules are allowed to bind to the surface. During drying, the molecules are straightened and uniformly stretched by the hydrodynamic action of the receding meniscus (Fig. 2). Molecules prepared in this manner are stretched remarkably homogeneously to approximately ~2.3 kb/μm, i.e., approximately 30% over the length predicted for a double stranded DNA molecule of the same size (50-53). We showed previously that cloned DNA fragments can readily be mapped by FISH onto DNA molecules prepared by molecular combing, and referring to its quantitative nature, we termed our technique 'Quantitative DNA Fiber Mapping' (QDFM; 51). We also showed that QDFM can be applied to DNA molecules larger than 1 Mbp, which allowed us to map probes with near

kilobase resolution onto whole yeast chromosomes and large YAC clones from the CEPH/Genethon library (52). Because the DNA fibers are easily accessible to probes and detection reagents, hybridization efficiencies are typically high and allow the routine detection of DNA targets as small as 500-1000 bp (51,54-55). In addition to the construction of high-resolution physical maps (56, 57), QDFM has proven useful in studies of DNA replication (58).

The present article provides an in-depth description of the experimental procedures that will allow FISH experts as well as the novice to apply QDFM successfully to their research projects. Three practical examples demonstrate the application of QDFM in genome research.

2. Materials

2.1 Cell Culture and DNA Isolation

1. The AHC medium is prepared by adding 36.7 g of AHC powder (BIO 101, Vista, CA) per liter water (*See* **Note 1**), autoclave at 121°C for 15 min.
2. AHC agar (BIO 101): add 53.7 g of AHC agar medium per liter of purified water. Autoclave at 121°C for 15 min. and cool to 50°C. Then, mix well and pour plates. Store plates upside down in plastic bags at 4°C.
3. Tris HCl (tris(hydroxymethyl)aminomethane) (*See* **Note 2**): prepare 1 M stock solutions in 500ml bottles, adjust pH to pH 7.5, 8.0 or 8.3 and autoclave.
4. Lysozyme (Sigma Chemicals, St.Louis, MO) is prepared as stock solution (50 mg/ml in 10 mM Tris, pH 7.5), and stored in 100-150 µl aliquots at -20°C. Once thawed, do not refreeze.
5. Zymolase (70000 U/g) is best prepared as 10 mg/ml stock in 50 mM KH_2PO_4, pH 7.8, 50% glycerol. Store at -20°C.
6. Luria Broth (LB) is prepared by dissolving 10 g of Luria-Bertani powder per 400 ml of purified water in a 500 ml bottle. Autoclave at 121°C for 15 minutes and allowed to cool for storage and use.
7. SCE buffer is 1 M sorbitol, 0.1 M Na citrate, 10 mM ethylenediamine tetraacetic acid (EDTA), pH 7.8.
8. The ES Buffer is 0.5 M EDTA (pH 8.0, Invitrogen, Gaithersburg, MD), 1% sarcosyl.
9. The TE (Tris/EDTA) buffer, 1x is 10mM Tris·HCl, 1 mM EDTA, pH 7.4, 7.5 or 8.0.; TE50 buffer is 10 mM Tris·HCl, 50 mM EDTA, pH 7.8, and 10x Tris/borate/EDTA (TBE) buffer is 890 mM Tris base, 890 mM boric acid, 20mM EDTA.

10. The alkaline lysis (AL) set for isolation of P1, PAC or BAC DNA is comprised of three solutions. The amounts listed here are sufficient for 12 isolations at a level of 20 ml cell culture. AL Solution I is 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl pH 8.0. Add 4 ml of 0.5 M glucose, 0.8 ml of 0.5 M EDTA and 1 ml of 1M Tris-HCl, pH 8.0 to 34.2 ml water. Store at 4°C. AL Solution II is 0.2 N NaOH, 1 % SDS. Add 1.4 ml of 10N NaOH, 7 ml of 10% SDS to 61.6 ml water. AL Solution III is 3 M NaOAc, pH 4.8 in water.
11. Plasmid and cosmid DNAs are isolated using commercially available kits such as the Geneclean II kit (BIO 101).

2.2 Functionalization of Glass Surfaces and Molecular Combing

1. Prepare a solution of 0.1% aminopropyltriethoxysilane (APS) in 95% ethanol just prior to use.
2. YOYO-1 (Invitrogen): stock is 1 mM in DMSO. Dilute 1:1000 with water prior to use. Store at -20°C and discard diluted dye after 1 week.

2.3 Preparation of DNA Probes

1. Gel Loading dye (6x) is 1% bromophenol blue in 30% glycerol.
2. Thermus aquaticus (Taq) DNA polymerase buffer (10x) has a concentration of 500 mM KCl, 100 mM Tris·HCl (made from 1 M Tris·HCl, pH 8.3), 15 mM $MgCl_2$.
3. Modified nucleotide mix (10x) for labeling in combination with 1 mM digoxigenin-11-dUTP, fluorescein isothiocyanate (FITC)-12-dUTP or other dTTP analogs is prepared by combining 5 µl each of 100 mM dATP, 100 mM dGTP and 100 mM

dCTP (Amersham) with 2.5 µl of 1 M Tris-HCL, pH 7.5, 0.5 µl 0.5 M EDTA, pH 8.0 (Invitrogen) and 232 µl water for a total of 250 µl. The final concentration of nucleoside triphosphates is 2 mM each. Store at -20°C.

2.4 FISH

1. The 20x SSC stock buffer is 3 M NaCl, 0.3 M Na_3·citrate·$2H_2O$, pH 7.0.
2. Denaturing solution is 70% formamide (FA; Invitrogen), 2xSSC, pH 7.0. Store at 4°C.
3. Hybridization Master Mix (MM2.1) is 14.3% (w/v) dextran sulfate, 78.6% FA, 2.9 X SSC, pH 7.0. For 10 ml MM2.1, mix 1.45 ml of 20X SSC with 0.7 ml water, dissolve 1.43 g dextran sulfate (Calbiochem, San Diego, CA), incubate overnight, then add 7.86 ml formamide (Invitrogen). Aliquot in 1.5 ml microcentrifuge tubes and store at -20°C.
4. Maleic acid buffer is 100 mM maleic acid, 150 mM NaCl; adjust to pH 7.5 with concentrated NaOH.
5. Blocking stock solution: dissolve blocking reagent (Roche Molecular Biochemicals) in 10% (w/v) maleic acid buffer with shaking and heating. Autoclave stock solution and store in aliquots at 4°C. For Slide Blocking Solution (5x SSC containing 2% Blocking Reagent and 0.1% N-lauroyl sarcosine) combine 0.05 g N-lauroyl sarcosine (Na salt) and 1 g Blocking Reagent with 12.5 ml of 20x SSC (pH 7.0), add 30ml water. Heat to 60°C while stirring and bring the final volume to 50ml with water, when the Blocking Reagent is dissolved. Aliquot into 1.5 ml tubes, spin at 2000 rpm for 10 min and store at 4°C.

6. PN buffer is 0.1M sodium phosphate, pH 8.0, 0.1% nonidet-P40 in water. This buffer is prepare by dissolving 26.8g of Na_2HPO_4 (dibasic) in 1l water and 8.2794g of Na_2HPO_4 (monobasic) in 600ml water. Note the amount of monobasic solution added. Tirate the dibasic solution (pH >9) by adding small volumes of the monobasic solution (pH ~4.5) until a pH of 8.0 is reached. Add Non-Idet P-40 to 0.05% (v/v).
7. PNM buffer: dissolve 5 g of non-fat dry milk (Carnation, Wilkes-Barre, PA) in 100 ml of PN buffer, incubate at 50°C overnight and add 1/50 volume sodium azide, spin at 1000 g for 30 min, aliquot clear supernatant into 1.5 ml tubes and store at 4°C. Spin at 2000 g for 30 sec prior to use of the clear supernatant.
8. Rhodamine-conjugated antibodies against digoxigenin, made in sheep (Roche Molecular Biochemicals), mouse-derived antibodies against FITC (DAKO, Carpintera, CA), FITC-conjugated anti-mouse antibodies made in horse (Vector Labs, Burlingame, CA), and biotinylated anti-avidin antibodies made in goat (Vector Labs) are prepared as stock solutions (1 mg/ml) in PNM, stored at 4°C, and diluted 1:50 with PNM just prior to use. Store at 4°C. Avidin conjugated to AMCA or FITC (Vector Labs): stock solution is 2 mg/ml in PNM, dilute 1:500 prior to use. Store at 4°C.
9. Antifade solution is 1% p-phenylenediamine, 15 mM NaCl, 1 mM H_2PO_4, pH 8.0, 90% glycerol. Store in 1ml aliquots at -80°C and keep one aliquot at -20°C for everyday use. (*See* **Note 3**).

2.5 Image Acquisition and Analysis and Construction of High-resolution Physical Maps

1. Quantitative DNA fiber mapping experiments require only standard laboratory equipment and access to a fluorescence microscope equipped with a film or electronic camera.
2. Filters are capable of excitation in single bands centered around 360nm, 405 nm, 490 nm, 555 nm, and 637 nm, and visualization in multiple bands in the vicinities of 460 nm (blue), 520 nm (green), 600nm (red) and 680nm (infrared) are desirable. Single color images are collected using a CCD camera (Xilix, Hamamatsu, Vosskuehler, Photometrics or similar) connected to a computer workstation (51). When images are recorded on film, standard film with a sensitivity of ASA400 is sufficient.

3. Methods

In QDFM, a small volume of isolated DNA molecules in aquous solution is placed on glass (51,53) or freshly cleaved sheets of mica (54) surface functionalized so that the majority of DNA molecules attach at one or both ends. The DNA solution is then spread over a larger area by placing a coverslip on top, and additional DNA molecules bind to the surface. During the subsequent drying step, molecules tethered to the support via either one or both ends (if linear) or via intermediate nicks in the double-stranded molecule (if the molecule is circular) are straightened and uniformly stretched by the hydrodynamic action of the receeding meniscus, i.e., the surface tension at the water-air interface.

3.1 Isolation of DNA for QDFM

1. DNAs are isolated from plasmid, cosmid, P1/PAC and BAC clones using either a commercially available purification kits or an alkaline lysis protocol (see 3.3.1 below). For the larger inserts, inserts are sized by pulse field gel electrophoresis (PFGE). Digestion of DNA with a rare cutting restriction enzyme produces linear high molecular weight DNA molecules, but the alkaline lysis procedure typically provides sufficient amounts of nicked circular or randomly broken DNA suitable for QDFM (52).
2. In general, the DNA is loaded on a 1.0% low melting point agarose (Biorad, Hercules, CA) gel and electrophoresed for about 15 hr. To efficiently separate DNA molecules of several hundred kb, we use a pulsed field gel electrophoresis system (Biorad).

3.1.1. Pulsed Field Gel Electrophoresis (PFGE)

1. The agarose plug preparation and pulsed field gel electrophoresis using a PFGE system (BioRad) follow standard protocols. Typically, a diluted solution of YACs in AHC medium is plated on AHC plates, and 5-15 individual YAC colonies are tested to account for deletions. In most cases, the largest clone carries the least deletion(s).
2. Preparation of gel plugs containing YACs (Invitrogen; stored at -80°C): spin down cells grown in 5 ml AHC media at 400 rpm for 6 min. Resuspend cells in 0.5 ml of 0.125 M EDTA, pH 7.8. Spin again and resuspend the cell pellet in 500 µl of SCE. Mix with an equal volume of 1.5% low melting point (LMP) agarose preheated to 43°C. Quickly pipet up and down, then vortex gently for 1-2 sec to mix. Pipet into plug molds (Biorad) and allow to solidify at room temperature or on ice.
3. Remove plugs from molds, incubate samples in 2 ml SCE containing 100 µl of zymolase and shake at 150 rpm at 30°C for 2.5 hrs to overnight. Replace SCE buffer with 2 ml of ES containing 100 µl of proteinase K (20 mg/ml, Roche). Shake 5 hrs to overnight at 50°C, and rinse plugs rinse 5 times with 6 ml of TE50 for 30 min each rinse. Store the plugs at 4°C until use.
4. PFGE running conditions for separation of YACs from yeast chromosomes are voltage gradient, 6 V/cm; switching interval, 79sec forward, 94sec reverse; running time, 38hrs; agarose concentration, 1.0% LMP agarose; running temperature, 14°C; running buffer, 0.5x TBE.
5. PFGE running conditions for separation of full length P1/PAC/BAC clones from debris are voltage gradient, 6 V/cm; switching interval, 2 sec forward, 12 sec reverse;

running time, 18 hrs; agarose concentration, 1.0% LMP agarose; running temperature, 14 °C; running buffer, 0.5x TBE (*See* **Note 4**).

6. For probe production and determination of optimal PFGE conditions: stain the gel with ethidium bromide (EB, 0.5 µg/ml in water), cut out a gel slice containing the target DNA band and transfer slice to a 14 ml polystyrene tube (cat.# AS-2264, Applied Scientific). Wash slice with water for 30 minutes, and then wash with 1x agarase buffer for 30 min.
7. For isolation of high molecular weight DNAs: run duplicate samples on the right and left side of the gel, respectively. After a predetermined run time, cut gel in half, and stain one half with EB. Measure the migrated distance on a UV transilluminator, cut out a gel slice at the corresponding position from the unstained half and proceed as described in 5.

3.1.2. Recovery of High Molecular Weight DNA from LMP Agarose Gel Slices

1. The DNA is recovered from the low melting point agarose slab gel by excising the appropriate bands using a knife or razor blade. High molecular weight DNA is then isolated by β-agarase digestion of the gel slices. Equilibrate gel slice in agarase buffer.
2. Melt the gel completely by incubating it for 10 minutes at 85°C, then transfer the molten agarose to a 43°C waterbath.
3. Add 1 µl β-agarase (New England Biolabs (NEB)) for every 25 µl of molten agarose and incubate at 43°C for 2 hrs.

4. Add an equal volume of 200 mM NaCl, and store the DNA samples at 4°C until use (*See* **Note 5**).

3.2 Pretreatment of Microscope Slides and Preparation of DNA Fibers on Glass

1. The derivatization of glass substrates is among the most critical steps of the procedure. The slides should have the capacity to bind DNA molecules at one or both ends, but allow the molecules to stretch during the subsequent drying.
2. Solid substrates for QDFM are prepared in batches of 20-50 by derivatization of glass microscope slides (See **Note 6**), coverslips or sheets of mica with APS resulting in primary amino groups on the glass surface (51, 53; See **Note 7**).
3. Clean glass slides mechanically by repeated rubbing with wet cheesecloth to remove dust and glass particles.
4. Rinse several times with water, immerse slides in boiling water for 10 minutes and air dry.
5. Immerse slides in 18 M sulfuric acid for at least 30 minutes to remove organic residues, followed by immersion in boiling water for 2 minutes.
6. Immerse precleaned dry slides in a solution of 0.1% aminopropyltriethoxysilane (APS) in 95% ethanol for 10 minutes.
7. Remove slides from the silane solution and rinse several times with water, and immerse in water for 2 minutes.
8. Dehydrate by immersing in absolute ethanol and dry slides upright for 10 minutes at 65°C on a hot plate.
9. Store slides for 2-6 weeks at 4°C in a sealed box under nitrogen prior to use.

3.2.1. Molecular Combing

1. In a typical experiment, 1-2 µl of clonal DNA are mixed with an equal amount of YOYO-1 (1 µM or 0.1 µM) and 8 ul water. One or two microliter of this diluted DNA is applied to an untreated coverslip, which is then placed DNA side down on the APS-derivatized slide.
2. The DNA concentration can be estimated in the fluorescence microscope using a filter set for FITC and adjusted as needed (*See* **Note 8,9**). As early as after two minutes of incubation at room temperature, the untreated coverslip can be removed slowly from one end, allowing the receeding meniscus to stretch the bound DNA molecules ('fibers') in one direction (53, 59) (*See* **Note 10**).
3. Alternatively, the slide or coverslip sandwich can be allowed to dry overnight at room temperature, after which the untreated coverslip is removed by lifting it on one side with a razor blade. Slides carrying DNA fibers are rinsed briefly with water, drained, allowed to dry at room temperature and 'aged' in ambient air at 20°C for one week before hybridization. Extra slides are stored at 4°C.

3.3 Preparation of DNA probes

A typical QDFM experiment uses several different probes simultaneously. One probe is needed to counterstain the DNA fibers. This probe is usually prepared by labeling DNA from the same batch that was used to prepare the fibers. Probes for sequences to be mapped along the DNA fibers are made such that they can be detected in a different color. Furthermore, it is recommended to include landmark

probes that provide reference points by binding specifically to the vector part or the ends of DNA molecules (60).

3.3.1. Alkaline Lysis Protocol and Purification of DNA from P1, PAC or BAC clones

1. The P1/PAC/BAC clones typically show far fewer deletions than YACs, so that it often suffices to pick 2-3 colonies from a plate, grow them overnight in AHC, and extract the DNA using an alkaline lysis protocol. The DNA can then be loaded directly onto the PFGE gel using a common gel loading dye. This protocol describes the isolation of DNA from ~20 ml overnight cultures using 40 ml Oak Ridge centrifugation tubes (Nalgene). The protocol can be scaled down to accommodate smaller volumes.
2. Grow cultures overnight in 25-30 ml LB medium containing the recommended amount of antibiotic.
3. Prepare Oak Ridge tubes. Write the clone ID on a small piece of tape sticked to the cap. Spin 18.5 ml of culture at 2000 g for 10 min at 4°C and discard the supernatant.
4. Resuspend the pellet in 2340 µl of AL Solution I, then add 100 µl of lysozyme stock to each tube. Incubate tubes for 5 min at room temperature. Then, place the tubes on ice.
5. Add 5.2 ml of AL Solution II. The mixture should now become clear. Mix gently by inverting the tubes several times. Incubate for 5 min on ice.
6. Add 3.8 ml of AL Solution III and mix gently by inverting the tubes several times. Incubate for 10 min on ice.
7. Spin for 15 min at 14,000 g.

8. Transfer 10.4 ml of supernatant into a new Oak Ridge tube, add 5.8 ml of isopropanol and mix gently by inverting tubes several times. Use the old cap (with the ID sticker) on the new tube.
9. Spin for 5 min at ~10,000 g and discard the supernatant. Watch the pellet!
10. Wash the pellet in cold 70% ethanol. Let the pellets dry briefly, i.e., at ~20-40 min at 20°C to 37°C.
11. Resuspend the pellet in 0.8 ml of TE buffer and split the volume into two 1.5 ml microcentrifuge tubes.
12. Add 400 µl phenol/chloroform/isoamyl alcohol (Invitrogen) to each tube. All centrifugations during the following phenol/chloroform extraction are done at 12,000 g.
13. Vortex for 15 sec and spin down for 3 min.
14. Remove most of the bottom layer and spin again for 3 min.
15. Transfer the top layer to new microcentrifuge tubes and add 400 µl chloroform/isoamyl alcohol (24:1, v/v; Invitrogen)
16. Vortex well for 15 sec, spin down for 3 min and remove most of the bottom layer followed by a second centrifugation for 3 min.
17. Transfer top layer to a new microcentrifuge tube, add 2.5 volumes, i.e., 1 ml of 100% ethanol and let the DNA precipitate for 30 min. at -20°C.
18. Spin down for 15 min, discard the supernatant and wash the pellet in ice cold 70% ethanol, spin again briefly, remove supernatant and air dry the pellet.
19. Resuspend the pellet in 20-40µl TE, pH 7.4 containing 10 µg/ml RNAse (Roche) made DNase-free by boiling at 100°C for 10 min and stored in aliquots at -20°C..
20. Incubate 30 min at 37°C (in water bath) and store at -20°C until use.

3.3.2. Preparation of DNA from Yeast Artificial Chromosome (YAC) Clones

1. Retrieve the desired yeast clone containing the YAC from the library and grow it on AHC agar for 2-3 days at 30°C. Pick colonies from the plate and culture the clones in up to 35 ml AHC media at 30°C for 2-3 days.
2. Centrifuge cells in AHC media at 2000 g at 4 °C for 5 min.
3. Decant the supernatant and resuspend cells in 3 ml total of 0.9 M sorbitol, 0.1 M EDTA, pH 7.5, containing 4 μl β-mercaptoethanol, followed by addition of 100 μl of zymolase (2.5 mg/ml), and incubate at 37°C for 60 min.
4. Pellet the cells at 2000 g and 4 °C for 5 min and decant supernatant.
5. Resuspend pellet in 5 ml of 50 mM Tris, pH 7.4, 20 mM EDTA. Add 0.5 ml of 10% SDS and mix gently. Incubate at 65 °C for 30 min.
6. Add 1.5 ml of 5 M potassium acetate and place on ice for 60 min.
7. Spin at 12,000 g for 15 min at 4 °C, and transfer the supernatant to a new tube.
8. Mix the supernatant gently with 2 volumes of 100% ethanol by inverting the tube a few times. Spin in 5000 rpm (2000 g) for 15 min at room temperature.
9. Prepare sets of four 1.5 ml microcentrifuge tubes for each clone.
10. Decant supernatant and air dry the pellet. Resuspend pellet in 3 ml of 1x TE, pH 7.5.
11. Transfer 750 μl of the DNA solution to each of the four 1.5 ml microcentrifuge tubes.
12. Add an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0), vortex well and spin at 10,000g for 3 min.
13. Transfer the top layer to new 1.5 ml microcentrifuge tubes and add an equal volume of chloroform/isoamyl alcohol (24:1). Vortex well and centrifuge at high speed (10,000 g) for 3 min.

14. Transfer the top layer to new 1.5 ml microcentrifuge tubes. Add 40 µl of RNAse (1 mg/ml, DNAse free) to each of the four tubes and incubate at 37°C for 30 min.
15. Add 1 volume of isopropanol and gently mix by inversion. Centrifuge at high speed (10,000 g) for 20 min.
16. Decant supernatant, wash pellet with 1 volume of cold 70% ethanol, and centrifuge at 10,000 g for 3 min.
17. Decant the 70% ethanol, air dry the pellet, and resuspend the pellet in 30 µl 1x TE.
18. The DNA concentration is measured after the pellet is completely dissolved.

3.3.3 Generation of Probes by in vitro DNA Amplification

1. In vitro DNA amplification using the polymerase chain reaction (PCR) is a very efficient method to synthesize probe DNA. It can be applied to amplify a particular DNA sequence, such as a part of the cloning vector (60), or with mixed-base primers to perform arbitrary amplification of virtually any sequence of interest (51,59,61). As illustrated in the following paragraphs, the former amplification can be applied to prepare DNA landmark probes, while the latter allows the preparation of probes to counterstain the fibers.
2. The generation of P1/PAC-, BAC- and YAC-vector probe DNA takes advantage of the access to published vector sequences. PCR primers are typically designed to amplify fragments of 1100-1400 bp of vector sequence (51,52,60). Various oligonucleotide pairs have been designed in several laboratories including ours and are used in either single pairs or combinations (51,52,56, 60). The PCR usually follows standard conditions, i.e., a Tris-HCl buffer containing 1.5 mM $MgCl_2$ and 1

unit Taq DNA polymerase per 50 µl reaction is used, annealing temperatures range from 50°C to 60°C.

3. The YAC cloning vectors pJs97 and pJs98, cloned in plasmid vectors (Invitrogen), can be used to prepare probes useful to determine the orientation of the YAC insert (56). For this purpose, plasmid DNA is extracted using the above alkaline lysis protocol or a commercial kit and labeled by random priming as described below.
4. The DNA probes for counterstaining the YAC DNA fibers are generated by mixed base oligonucleotide primed PCR (also referred to as degenerate oligonucleotide primed PCR or 'DOP-PCR'; 61,62). An aliquot of the HMW DNA obtained by PFGE for fiber preparation is PCR amplified for a total of 42 cycles with oligonucleotide primers that anneal about every 200-800 nucleotides. In our preferred scheme, we use two different DNA amplification programs (62). Initially we perform a few manual PCR cycles using T7 DNA polymerase to extend the oligonucleotide primers at a relatively low temperature. Next, DNA copies prepared in these first cycles are amplified using the thermostable Taq DNA polymerase and a rapid thermal cycling scheme.
5. In the first amplification stage, T7 DNA polymerase ('Sequenase II', Amersham) is used in 5-7 cycles to extend the mixed base primer JUN1 (5'-CCAAGCTTGCATGCGAATTCNNNNCAGG-3', N=ACGT) that is annealed at low temperature. Briefly, 2-3 µl of high molecular weight DNA solution (after PFGE purification) are removed from the bottom of each tube and PCR amplified using the following conditions: denaturation at 92°C for 3 min, primer annealing at 20°C for 2 min and extension at 37°C for 6 min. Sequenase II enzyme must be added after

each denaturation.

6. In the second amplification stage, 10 µl of the reaction products are resuspended in a 200µl Taq DNA amplification buffer and amplified with primer JUN15 (5'-CCCAAGCTTGCATGCGAATTC-3') with the following PCR conditions: denaturation at 94°C for 1 min, primer annealing at 50°C for 1 min, and extension at 72° for 2 min, repeated for 30 cycles. After precipitation of the PCR products in 1.2 vol of isopropanol, the products are resuspended in 30 µl of TE buffer. Subsequently, 1.5 µl of this solution is labeled in a 25 µl random priming reaction incorporating digoxigenin-11-dUTP or FITC-12-dUTP.
7. DNA amplification is confirmed by electrophoresing a 5 ul aliquot on a 3% agarose gel in TBE buffer containing 0.5 µg/ml ethidium bromide.

3.3.4. Probe Labeling via Random Priming and Hybridization

1. Labeling of DNA by random priming is a reliable method and, in our laboratory, is applied routinely to label DNA fragments from 100bp to several hundred kb. The procedure involves an initial thermal denaturation of the DNA to allow the random oligonucleotides (‘primers’) to anneal. Thus, restriction or hydrolysis of large molecules is not necessary. Several companies now offer kits for random priming reactions. Slight differences exist with regard to enzyme activity, amount of random primers and cost per reaction.
2. The concentration of PCR products can be estimated from the agarose gels run to confirm target amplification. If a sufficient amount of clonal or genomic DNA is available, one or two microliters can be used to accurately determine the

concentration using Hoechst 33258 fluorometry using a TK100 fluorometer (Pharmacia).

3. Add 250 ng of DNA to water to a final volume of 7 μl in a 0.5 ml microcentrifuge tube.

4. Boil DNA at 100 °C for 5 min, then quickly chill on ice.

5. For labeling with either digoxigenin-dUTP or FITC-dUTP, add:

2.5 μl	10x Modified Nucleotide Mixture
3.25 μl	1 mM dTTP
1.75 μl	digoxigenin-11-dUTP or FITC-12-dUTP (1 mM each, Roche)
10 μl	2.5X Random primers (BioPrime kit, Invitrogen)

6. For labeling the DNA with biotin, add 2.5 μl 10x dNTP mix provided with the BioPrime kit (containing biotin-14-dCTP), 5 μl water, and 10 μl 2.5x random primers).

7. Mix well, add 0.5μl DNA polymerase I Klenow fragment (40 units/μl, Invitrogen) and incubate in a waterbath at 37 °C for 120 min.

8. Add 2.5 μl of 10X stop buffer (Invitrogen, part of the BioPrime kit).

9. Store probe at –20 °C until use.

3.4 FISH

1. The hybridization procedure is very similar to protocols to used with metaphase spreads. In the hybridization mix, combine 1 μl of each probe, 1 μl of human COT1™ DNA (1 μg/μl, Invitrogen, optional), 1 μl of salmon sperm DNA (5 Prime – 3 Prime, Boulder, CO), and 7 μl of MM2.1.

2. Fiber hybridizations include a comparatively low concentration of a biotin- or FITC-labeled DNA probe prepared from the high molecular weight DNA that is used to prepare the fibers. This counterstain highlights the otherwise invisible DNA fibers and allows competitive displacement by the probes to be mapped along the DNA fiber (51,56). Additionally, one or several cloning vector-specific probes are included to allow a determination of the orientation of the insert (60).
3. Apply the hybridization mixture to the slides and coverslip. Avoid bubbles; if bubbles occur, try to squeeze them out gently with fine tip foreceps avoiding to move the coverslip.
4. Transfer the slides to a dry bath (or 'hot plate') and denature the DNA at 88°C for 90 sec.
5. Transfer the slides to a moisture chamber (a plastic or stainless steel box with a wet paper towel at the bottom and support such as cut disposable plastic pipettors to raise the slide) and incubate it overnight at 37 °C.
6. Wash and detection steps are not much different from protocols used for FISH to interphase and metaphase cells and have been described in detail (51,54). After hybridization, the slides are washed three times in 2x SSC at 20°C for 10min each, then incubated with 100μl PNM buffer or blocking stock solution under a plastic coverslip at 20°C for 5 min (*See* **Note 11**). The slides are then incubated at room temperature for 30 min with 100 μl PNM buffer containing AMCA-avidin (Pharmacia), anti-digoxigenin-rhodamine (Roche) and a mouse antibody against FITC (DAKO) (*See* **Note 12**).
7. The slide is then washed two to three times in 2x SSC for 15 min each at 20 °C with

constant motion on a shaking platform.

8. If necessary, signals are amplified using a biotinylated antibody against avidin raised in goat (Vector) followed by another layer of AMCA-avidin, a Texas Red-labeled antibody against sheep raised in rabbit and a horse-anti-mouse antibody conjugated to FITC (Vector) (52).

9. The slide is mounted in 8 µl of antifade solution and covered by a 22 mm x 22 mm coverslip.

3.5 Digital Image Acquisition and Analysis and Map Assembly

1. Although not a prerequisite for QDFM, digital image acquisition and computer-assisted analysis greatly facilitate the quantitative analysis of hybridization images. Since QDFM is based on simple measurements of distances between probe hybridization domains, the analysis can alternatively be performed on images recorded on film and either printed or projected on a screen.
2. Images are acquired using a standard fluorescence microscope (Zeiss Axioskop or similar) equipped with 63x, 1.25 N.A. and 40x, 1.2 N.A. objectives, and a filter set for excitation and sinultaneous observation of DAPI, Texas Red/rhodamine, FITC and CY5 fluorescence, respectively (ChromaTechnology, Brattleboro, VT) (*See* **Note 13,14**).
3. For determination of map positions, interactive software is available for either Apple Macintosh, IBM/PC or SUN computers that allows the user to trace DNA fibers by drawing a straight or segmented line and then calculates the length of the line in pixels (52,56). The pixel spacing for the camera and the microscope objective used

in the experiment (a 63x objective is used for molecules up to 100 kb, a 40x objective for larger molecules) is known and is converted into μm or into kb using the factor of 2.3 kb/μm (51). After measuring all relevant distances along the DNA fibers in triplicate, the results in form of lists are then imported into Microsoft Excel spreadsheets and used to calculate average values for each fiber and mean values and standard deviations for individual experiments.

3.5.1 Construction of High-resolution Physical Maps

1. QDFM can facilitate the construction of high-resolution physical maps comprised of any combination of cosmid, P1, PAC or BAC clones in two ways: if a low resolution map is available, for example, in the form of a YAC contig, individual clones can be mapped directly onto DNA fibers prepared from the larger clones (3,51). Alternatively, a high resolution map can be constructed by measuring the extent and orientation of overlap between individual clones by hybridizing one clone onto another (Fig. 1). In most experiments, the approach taken will depend on the sources of the clones and might combine both schemes.

2. Figure 3 shows the mapping of a P1 clone (~81 kb insert, red) onto a colinear YAC clone (green). Precise localization of the region of overlap and measurement of distances from the ends of the YAC (distances 'A' and 'B') are facilitated by probes that mark specifically the ends of the YAC molecules (red, arrows).

3. The DNA fiber-based mapping of two P1 clones ('1107' and '1143') onto the colinear YAC clone '141G6' (~ 490 kb; 5) and determination of the size of the gap between these P1 clones by QDFM has been described (51; Fig. 4). Briefly, the degree and

uniformity of stretching achieved for the YAC molecules was assessed by measuring the lengths of the domains produced by hybridization with DNA from the ~81 kb P1 '1143' along 10 YAC fibers. The length of '1143' along the YAC fibers was 34.5 μm ± 2.55 μm, corresponding to a stretching of 2.3 kb/μm, almost identical to that achieved for lambda phage (51; Fig. 4C). This suggested that the degree of stretching is highly reproducible and independent of the length of the combed molecule.

4. The location of the P1 clone '1143' along the YAC was determined by measuring the distance of its hybridization signal from the marked end of the YAC (Fig. 4B,C). The hybridization domain of clone '1143' began at 49.2 μm or 114 kb (±5.7 kb, N=10) from the proximal end of the YAC and extended 81 kb, assuming a conversion factor of 2.3 kb/μm. Measurement of the hybridization domain of clone '1107' suggested a mean size of 89.9 kb (± 8.2 kb), which agrees well with the size of 88 kb obtained by PFGE analysis. This conversion also allowed us to estimate the size of the YAC as 496 kb (s.d. 37 kb, N= 4). This was in good agreement with published values ranging from 430 kb to 495 kb (5,29).
5. The extent of the gap between clones '1143' and '1107' (Fig. 4A) was found to be 10.9 μm or 25.4 kb (±1.1 kb) by measuring the physical distances between the P1 hybridization signals on 10 YAC fibers (51). Partial fibers showing hybridization signals along the gap region and part of the flanking P1's were sufficient for determining the size of the gap region since these all appeared to be equally stretched.

3.5.2 Quality Control of Individual DNA Sequencing Templates

1 The P1 clone #39 maps to the long arm of chromosome 20 band q13 (63). Sequencing templates were prepared by cloning size selected ~3 kb fragments from sonicated P1 #39 DNA into the plasmid vector pOT2 (63).

2. Digoxigenin-labeled probes prepared by random priming of plasmid DNA from eight sequencing templates (plasmid clones) were mapped onto DNA fibers prepared from Not 1-linearized molecules of P1 #39. The hybridization mixture contained the plasmid probe, 100 ng/μl human COT1 DNA (Invitrogen) to block hybridization of DNA repeat sequences, biotinylated P1 #39 DNA (here visualized in blue) and an FITC-labeled probe for the P1 vector (green). The bound plasmid DNA was visualized in red (Fig. 5). Plasmid probes that showed hybridization to vector sequences were also mapped by hybridization to DNA fibers prepared from an empty recombinant P1 (Genome Systems, St. Louis, MO). Figure 5 shows representative images of hybridized DNA fibers using six different plasmid probes. The map position of the ~3 kb plasmids was readily visible and is indicated in the black-and-white reproduction by an arrow (Fig. 5).

3. Map positions relative to the Sal 1 site (near the Sp6 promotor) of the P1 vector pAd10SacBII are obtained from QDFM images. Plasmid clones 9-d4, 2-a2, 4-c11, 4-h3 and 10-h8 were found to map exclusively to the insert of P1 #39, while clones 1c7 and 4-h5 hybridized to the insert as well as vector regions (Fig. 5). Gel electrophoretic analysis indicated that clones 1-c7 and 4-h5 were significantly larger than the other clones (Table I). Clone 1-c7 mapped to the insert adjacent to the unique Not 1 (T7) site at approximately the same position as clone 1-h3, but showed an additional hybridization signal adjacent to the Sal 1 site (not shown). Clone 4-h5

hybridized to the insert approximately 52 kb +/- 4 kb from the Sal I site, and showed a second hybridization signal of approximately 3 kb centered between positions 4500 and 7500 of the 16 kb pAd10SacBII vector (Fig. 5, arrowhead)(Genbank accession # U09128; Table I).

3.5.3. Localizing Contigs Comprised of ~3 kb Plasmid Clones

1. QDFM can map and orient contigs comprised of ~3 kb sequencing templates onto P1 or BAC molecules with a precision of a few kb.
2. The P1 clone #30 maps in roughly the same region of chromosome 20q13 as P1 #39 (3). The Fig. 6A shows the mapping of a 5 member contig (contig3) onto an Sfi I linearized P1 DNA molecule (clone #30). While this P1 clone was prepared to be sequenced at the LBNL Human Genome Center, we mapped onto it three plasmid contigs. Each contig was comprised of five to seven plasmid clones of approximately 3 kb in length (sequencing templates). QDFM let us measure the contig map position and extent, as well as the overlap between contigs with an accuracy of ~1 kb. The results (summarized in Fig. 6B) showed that contigs 2 and 3 overlap about 11 kb, but neither of them overlapped with contig 1. Thus, either of these two contigs can be sequenced in parallel with the non-overlapping contig 1.
3. The information regarding contig position will also facilitate the preparation of sequencing templates comprised of large insert DNA clones. In genomic regions, for which only shallow coverage exists, a minimal tiling path typically contains clones with extensive overlap. QDFM allows elimination of ~3 kb plasmid contigs that fall in overlapping regions. These investigations can be performed rapidly on either circular, enzymatically restricted or randomly broken DNA molecules.

4. Notes

1. Unless stated otherwise, all solutions should be prepared with purified water having a restistivity of 18.2 MΩ-cm and total organic content of less than five parts per billion. This standard is referred to as “water“ in this protocol.
2. Unless noted otherwise, all chemical were purchased from Sigma.
3. Allow the antifade solution to warm up for only a short period of time, remove an aliquot, then place it back into the freezer. Fresh antifade solution is colorless. Discard aliquot when solution has turned dark.
4. DNA isolated from P1, PAC or BAC clones can be loaded directly into the wells of the PFGE gel. Use 1μl of loading buffer per 5 μl of DNA solution.
5. The integrity of DNA molecules can be assessed by microscopic inspection of aliquots of DNA stained with 0.5 μM YOYO-1, before high molecular weight DNAs are used for DNA fiber preparation or stored at 4°C in 100 mM NaCl.
6. Slides from different manufacturers or the same brand, but different batches may produce different qualities of DNA fibers. Purchase a sufficiently large batch of slides from one manufacturer such as Erie Scientific (Portsmouth, NH), a supplier of glass slides which are sold under Fisher or BD labels. Avoid slides that are painted on one end, since the paint might dissolve during pretreatment. Preferable are slides that have a sandblasted or etched area at one end.
7. Coverslip silanation is performed similar to the procedure described for slides. Briefly, coverslips are rinsed with distilled water and dehydrated in 100% ethanol. Coverslips are derivatized with a 0.05–0.1 % solution of APS in 95% ethanol for 2 minutes. Coverslips are then rinsed and dried as described in section 3.2.

8. Binding of DNA molecule ends to the substrate and the stretching effect can be monitored in the fluorescence microscope by staining the DNA with 0.2-0.5μM YOYO-1 prior to deposition. This allows to exclude batches of slides that bind DNA too tightly. Dilute the YOYO-1 in water, since the antifade solution prevents the DNA molecules from binding.
9. The density of DNA molecules after DNA fiber stretching can be adjusted by altering the concentration of the DNA molecules prior to binding. Figure 2D-F shows the typical density of hybridized lambda DNA molecules. In experiments depositing lambda, P1, PAC or BAC DNA molecules, the fraction of intact DNA molecules can reach ~50-70%. Mapping can utilize both linear and circular DNA molecules. While binding of DNA molecules in their circular form helps to maintain their integrity, it interferes with DNA fiber stretching, and the molecules are found to be stretched to varying degrees. Mapping onto circular molecules can thus be used for rough estimation of overlap, and mapping on linear fibers for high precision measurements. This can be done in a single experiment, because some circular DNA molecules are sheared during deposition, thus providing randomly broken linear DNA molecules (see reference 53 for a more detailed description).
10. Different procedures have been described to stretch DNA molecules (47-51). In our hands, stretching involving a hydrodynamical force (meniscus) at 20°C or 4°C has proven most reproducible. There is, however, no need to wait until the preparation has dried to completion. Once the DNA molecules have bound to the substrate, the coverslip can be lifted to exert the hydrodynamic stretching force (53).

11. We found that even short periods of drying out of slides or part of them during the immunocytochemical signal amplification lead to unaccaptable levels of background fluorescence due to unspecific binding of detection reagents. It is important to just drain the liquids from the slides, and then rapidly apply the next solution, a blocking solution or antibodies.
12. If only two labels are used, i.e., biotin and digoxigenin, bound probes are detected with avidin-FITC DCS (Vector) and antidigoxigenin-rhodamine, respectively.
13. Most fluorochromes fade quickly. Under the microscope, we always minimize the exposure of slides to the excitation light. The key issue to good images is to use the DNA counterstain or one fiber probe to quickly localize areas showing a sufficiently high density of well strechted molecules, then switch to the image acquisition mode.
14. Always measure additional segments of the molecule such as the vector segment since these might provide additional information about the extent and homogeneity of DNA stretching.

Acknowledgements

This work was supported by a grant from the Director, Office of Energy Research, Office of Health and Environmental Research, U.S. Department of Energy, under contract DE-AC-03-76SF00098, the LBNL/UCSF Training Program in Genome Research sponsored by the University of California Systemwide Biotechnology Research and Education Program and NIH Grants CA80792 and HD45736.

References

1. Collins, F. and Galas, D. (1993) A new five-year plan for the U.S. human genome project. Science 262, 43-46.

2. Boguski, M., Chakravarti, A., Gibbs, R., Green, E., and Myers, R.M. (1996) The end of the beginning: the race to begin human genome sequencing. Genome Res. 6, 771-772.

3. Cheng, J.-F. and Weier, H.-U.G. (1997) Approaches to high resolution physical mapping of the human genome. In: Biotechnology International, C. F. Fox, T.H. Connor (eds.), Universal Medical Press, San Francisco, pp.149-157.

4. Martin J, Han C, Gordon LA, Terry A, Prabhakar S, She X, Xie G, Hellsten U, Chan YM, Altherr M, Couronne O, Aerts A, Bajorek E, Black S, Blumer H, Branscomb E, Brown NC, Bruno WJ, Buckingham JM, Callen DF, Campbell CS, Campbell ML, Campbell EW, Caoile C, Challacombe JF, Chasteen LA, Chertkov O, Chi HC, Christensen M, Clark LM, Cohn JD, Denys M, Detter JC, Dickson M, Dimitrijevic-Bussod M, Escobar J, Fawcett JJ, Flowers D, Fotopulos D, Glavina T, Gomez M, Gonzales E, Goodstein D, Goodwin LA, Grady DL, Grigoriev I, Groza M, Hammon N, Hawkins T, Haydu L, Hildebrand CE, Huang W, Israni S, Jett J, Jewett PB, Kadner K, Kimball H, Kobayashi A, Krawczyk MC, Leyba T, Longmire JL, Lopez F, Lou Y, Lowry S, Ludeman T, Manohar CF, Mark GA, McMurray KL, Meincke LJ, Morgan J, Moyzis RK, Mundt MO, Munk AC, Nandkeshwar RD, Pitluck S, Pollard M, Predki P, Parson-Quintana B, Ramirez L, Rash S, Retterer J, Ricke DO, Robinson DL, Rodriguez A, Salamov A, Saunders EH, Scott D, Shough T, Stallings RL, Stalvey M, Sutherland RD, Tapia R, Tesmer JG, Thayer N, Thompson LS, Tice H, Torney DC, Tran-Gyamfi

M, Tsai M, Ulanovsky LE, Ustaszewska A, Vo N, White PS, Williams AL, Wills PL, Wu JR, Wu K, Yang J, Dejong P, Bruce D, Doggett NA, Deaven L, Schmutz J, Grimwood J, Richardson P, Rokhsar DS, Eichler EE, Gilna P, Lucas SM, Myers, R.M., Rubin, E.M., and Pennacchio, L.A. (2004) The sequence and analysis of duplication-rich human chromosome 16. Nature 4, 988-994.

5. Chumakov, I., Rigault, P., Guillou, S., Ougen, P., Billault, G., Guasconi, S., Gervy, P., Le Gall, I., Soularue, P., Grinas, P., Bougueleret, L., Bellanné-Chantelot, C., Lacroix, B., Barillot, E., Gesnouin, P., Pook, S., Vaysseix, G., Frelat, G., Schmitz, A., Sambucy, J.L., Bosch, A., Estivill, X., Weissenbach, J., Vignal, A., Riethman, H., Cox, D., Patterson, D., Gardiner, K., Masahira, H., Sahaki, Y., Ichikawa, H., Ohsi, M., Le Paslier, D., Heilig, R., Antonorakis, S., Cohen, D. (1992) Continuum of overlapping clones spanning the entire human chromosome-21q. Nature 359, 380-387.
6. Cohen, D., Chumakov, I., and Weissenbach, J. (1993) A first-generation physical map of the human genome. Nature 366, 698-701.
7. Olson, M.V. (1993) The human genome project. Proc. Natl. Acad. Sci. U.S.A. 90:4338-4344.
8. Selleri, L., Eubanks, J.H., Giovannini, M., Hermanson, G.G., Romo, A., Djabali, M., Maurer, S., Mc Elligott, D.L., Smith, M.W., and Evans, G.A. (1992) Detection and characterization of "chimeric" yeast artificial chromosome clones by fluorescent in situ suppression hybridization. Genomics 1, 536-541.
9. Vetrie, D., Bobrow, M., and Harris, A. (1993) Construction of a 5.2-megabase physical map of the human X chromosome at Xq22 using pulsed-field gel electrophoresis and yeast artificial chromosomes. Genomics 15, 631-642.

10. Trask, B., Pinkel, D., and van den Engh, G. (1989) The proximity of DNA sequences in interphase cell nuclei is correlated to genomic distance and permits ordering of cosmids spanning 250 kilobase pairs. Genomics 5, 710-717.

11. Brandriff, B.F., Gordon, L.A., Tynan, K.T., Olsen, A.S., Mohrenweiser, H.W., Fertitta, A., Carrano, A.V., and Trask, B.J. (1992) Order and genomic distances among members of the carcinoembryonic antigen (CEA) gene family determined by fluorescence in situ hybridization. Genomics 12, 773-779.

12. Warrington, J.A. and Bengtsson, U. (1994) High-resolution physical mapping of human 5q31-q33 using three methods: Radiation hybrid mapping, interphase fluorescence in situ hybridization, and pulse field gel electrophoresis. Genomics 24, 395-398.

13. Lu-Kuo, J.M., Le Paslier, D., Weissenbach, J., Chumakov, I., Cohen, D., and Ward, D.C. (1994) Construction of a YAC contig and a STS map spanning at least seven megabasepairs in chromosome 5q34-35. Hum. Mol. Genet. 3, 99-106.

14. Green, E.D. and Olson, M.V. (1990) Systematic screening of yeast artificial-chromosome libraries by use of the polymerase chain reaction. Proc. Natl. Acad. Sci. U.S.A. 87, 1213-1217.

15. Coffey, A.J., Roberts, R.G., Green, E.D., Cole, C.G., Butler, R., Anand, R., Giannelli, F., and Bentley, D.R. (1992) Construction of a 2.6-Mb contig in yeast artificial chromosomes spanning the human dystrophin gene using an STS-based approach. Genomics 12, 474-484.

16. Weissenbach, J., Gyapay, G., Dib, C., Vignal, A., Morissette, J., Millasseau, P., Vaysseix, G., and Lathrop, M. (1992) A second-generation linkage map of the human genome. Nature 359, 794-801.

17. Locke, J., Rairdan, G., McDermid, H., Nash, D., Pilgrim, D., Bell, J., Roy, K., and Hodgetts, R. (1996) Cross-screening: A new method to assemble clones rapidly and unambiguously into contigs. Genome Res 6, 155-165.

18. Cai, W., Aburatani, H., Stanton, V.P., Housman, D.E., Wang, Y.K., and Schwartz, D.C. (1995) Ordered restriction endonuclease maps of yeast artificial chromosomes created by optical mapping on surfaces. Proc. Natl. Acad. Sci. U.S.A. 92, 5164-5168.

19. Samad, A., Huff, E.J., Cai, W., and Schwartz, D.C. (1995) Optical Mapping: a novel, single-molecule approach to genomic analysis. Genome Res 5, 1-4.

20. Waterston, R. and Sulston, J. (1995) The genome of Caenorhabditis elegans. Proc. Natl. Acad. Sci. USA 92, 10836-10840.

21. Bellanné-Chantelot, C., Lacroix, B., Ougen, P., Billault, A., Beaufils, S., Bertrand, S., Georges, I., Glibert, F., Gros, I., Lucotte, G., Susini, L., Codani, J.-J., Gesnouin, P., Pook, S., Vaysseix, G., Lu-Kuo, J., Ried, T., Ward, D., Chumakov, I., Le Paslier, D., Barillot, E., and Cohen, D. (1992) Mapping the whole human genome by fingerprinting yeast artificial chromosomes. Cell 70, 1059-1068.

22. Zucchi, I. and Schlessinger, D. (1992) Distribution of moderately repetitive sequences pTR5 and LF1 in Xq24-q28 human DNA and their use in assembling YAC contigs. Genomics 12, 264-275.

23. Porta, G., Zucchi, I., Hillier, L., Green, P., Nowotny, V., D'Urso, M., and Schlessinger, D. (1993) Alu and L1 sequence distributions in Xq24-q28 and their comparative utility in YAC contig assembly and verification. Genomics 16, 417-425.

24. Bell, C., Budarf, M.L., Nieuwenhuijsen, B.W., Barnoski, B.L., and Buetow, K.H. (1995) Integration of physical, breakpoint and genetic maps of chromosome 22. Localization of 587 yeast artificial chromosomes with 238 mapped markers. Hum. Mol. Genet. 4, 59-69.

25. Foote, S., Vollrath, D., Hilton, A., and Page, D.C. (1992) The human Y chromosome: overlapping DNA clones spanning the euchromatic region. Science 258, 60-66.

26. Stallings, R.L., Doggett, N.A., Callen, D., Apostolou, S., Chen, L.Z., Nancarrow, J.K., Whitmore, S.A., Harris, P., Michison, H., Breuning, M., Saris, J.J., Fickett, J., Cinkosky, M., Torney, D.C., Hildebrand, C.E., and Moyzis, R.K. (1992) Evaluation of a cosmid contig physical map of human chromosome 16. Genomics 13, 1031-1039.

27. Tynan, K., Olsen, A., Trask, B., de Jong, P., Thompson, J., Zimmermann, W., Carrano, A.V., and Mohrenweiser, H. (1992) Assembly and analysis of cosmid contigs in the CEA-gene family region of human chromosome 19. Nucleic Acids Res. 20, 1629-1636.

28. Nizetic, D., Gellen, L., Hamvas, R.M., Mott, R., Grigoriev, A., Vatcheva, R., Zehetner, G., Yaspo, M.L., Dutriaux, A., Lopes, C., Delabar, J.-M., Van Broeckhoven, C., Potier, M.-C., and Lehrach, H. (1994) An integrated YAC-overlap and 'cosmid-pocket' map of the human chromosome 21. Hum.Mol.Genet. 3, 759-770.

29. Patil, N., Peterson, A., Rothman, A., DeJong, P.J., Myers, R.M., and Cox, D.R. (1994) A high resolution physical map of 2.5 Mbp of the Down syndrome region on chromosome 21.Hum.Mol.Genet. 3, 1811-1817.

30. Cherry, J.M., Ball, C., Weng, S., Juvik, G., Schmidt, R., Adler, C., Dunn, B., Dwight, S., Riles, L., and Mortimer, R.K. (1997) Genetic and physical maps of Saccharomyces cerevisiae. Nature 387(Suppl), 67-73.

31. Pierce, J.C., Sauer, B., and Sternberg, N. (1992) A positive selection vector for cloning high molecular weight DNA by the bacteriophage-P1 system - improved cloning efficacy. Proc. Natl. Acad. Sci. U.S.A. 89, 2056-2060.

32. Ioannou, P.A., Amemiya, C.T., Garnes, J., Kroisel, P.M., Shizuya, H., Chen, C., Batzer, M., and DeJong, P.J. (1994) A new bacteriophage P1-derived vector for the propagation of large human DNA fragments. Nature Genet. 6, 84-89.

33. Shizuya, H., Birren, B., Kim, U.J., Mancino, V., Slepak, T., Tachiiri, Y., and Simon, M. (1992) Cloning and stable maintenance of 300-kilobase-pair fragments of human DNA in Escherichia coli using an F-factor-based vector. Proc. Natl. Acad. Sci. U.S.A. 89, 8794-8797.

34. Branscomb, E., Slezak, T., Pae, R., Galas, D., Carrano, A.V., and Waterman, M. (1990) Optimizing restriction fragment fingerprinting methods for ordering large genomic libraries. Genomics 8, 351-366.

35. Nelson, D.L. (1991) Applications of polymerase chain reaction methods in genome mapping. Current Opinion Genet. Devel. 1, 62-68.

36. Chang, E., Welch, S., Luna, J., Giacalone, J., and Francke, U. (1993) Generation of a human chromosome 18-specific YAC clone collection and mapping of 55 unique YACs by FISH and fingerprinting. Genomics 17, 393-402.

37. Riles, L., Dutchik, J.E., Baktha, A., McCauley, B.K., Thayer, E.C., Leckie, M.P., Braden, V.V., Depke, J.E., and Olson, M.V. (1993) Physical maps of the six smallest chromosomes of Saccharomyces cerevisiae at a resolution of 2.6-kilobase pairs. Genetics 134, 81-150.

38. Gillett, W., Hanks, L., Wong, G.K., Yu, J., Lim, R., and Olson, M. (1996) Assembly of high-resolution restriction maps based on multiple complete digests of a redundant set of overlapping clones. Genomics 33, 389-408.

39. Hoheisel, J.D. and Lehrach, H. (1993) Use of reference libraries and hybridisation fingerprinting for relational genome analysis. Febs Letters 325, 118-122.

40. Locke, J., Raidan, G., McDermid, H., Nash, D., Pilgrim, D., Bell, J., Roy, K., and Hodgetts, R. (1996) Cross-screening: A new method to assemble clones rapidely and unambiguously into contigs. Gen. Res. 6, 155-165.

41. Sapolsky, R.J. and Lipshutz, R.J. (1996) Mapping genomic libray clones using oligonucleotide arrays. Genomics 33, 445-456.

42. Green, E.D., Mohr, R.M., Idol, J.R., Jones, M., Buckingham, J.M., Deaven, L.L., Moyzis, R.K., and Olson, M.V. (1991) Systematic generation of sequence-tagged sites for physical mapping of human chromosomes application to the mapping of human chromosome 7 using yeast artificial chromosomes. Genomics 11, 548-564.

43. Arveiler, B. (1994) Yeast artificial chromosome recombinants in a global strategy for chromosome mapping. Amplification of internal and terminal fragments by PCR, and generation of fingerprints. Meth. Mol. Biol. 29, 403-423.

44. Aburatani, H., Stanton, V.P., and Housman, D.E. (1996) High-resolution physical mapping by combined Alu-hybridization/PCR screening: contruction of a yeast artificial chromosome map covering 31 centimorgans in 3p21-p14. Proc. Natl. Acad. Sci. USA 93, 4474-4479.

45. Cox, D.R., Burmeister, M., Price, E.R., Kim, S., and Myers, R.M. (1990) Radiation hybrid mapping: a somatic cell genetic method for constructing high-resolution maps of mammalian chromosomes. Science 250, 245-250.

46. Mungall, A.J., Edwards, C.A., Ranby, S.A., Humphray, S.J., Heathcott, R.W., Clee, C.M., East, C.L., Holloway, E., Butler, A.P., Langford, C.F. et al. (1996) Physical mapping of chromosome 6: a strategy for the rapid generation of sequence-ready contigs. DNA Sequence 7, 47-49.

47. Haaf, T. and Ward, D.C. (1994) High resolution ordering of YAC contigs using extended chromatin and chromosomes. Hum. Mol. Genet. 3, 629-633.

48. Florijn, R.J., Bonden, L.A.J., Vrolijk, H., Wiegant, J., Vaandrager, J.-W., Baas, F., den Dunnen, J.T., Tanke, H.J., van Ommen, G.-J.B., and Raap, A.K. (1995) High-resolution DNA Fiber-FISH for genomic DNA mapping and colour bar-coding of large genes. Hum. Mol. Genet. 4, 831-836.

49. Heiskanen, M., Karhu, R., Hellsten, E., Peltonen, L., Kallioniemi, O.P., and Palotie, A. (1994) High resolution mapping using fluorescence in situ hybridization to extended DNA fibers prepared from agarose-embedded cells. Biotechniques 17, 928-933.

50. Bensimon, A., Simon, A., Chiffaudel, A., Croquette, V., Heslot, F., and Bensimon, D. (1994) Alignment and sensitive detection of DNA by a moving interface. Science 265, 2096-2098.

51. Weier, H.-U.G., Wang, M., Mullikin, J.C., Zhu, Y., Cheng, J.-F., Greulich, K.M., Bensimon, A., and Gray, J.W. (1995) Quantitative DNA fiber mapping. Hum. Mol. Genet. 4, 1903-1910.

52. Wang, M., Duell, T., Gray, J.W., and Weier, H.-U.G. (1996) High sensitivity, high resolution physical mapping by fluorescence in situ hybridization on to individual straightened DNA molecules. Bioimaging 4, 1-11.

53. Hu, J., Wang, M., Weier, H.-U. G., Frantz, P., Kolbe, W., Olgletree, D. F., and Salmeron, M. (1996) Imaging of single extended DNA molecules on flat (aminopropyl)triethoxysilane-mica by atomic force microscopy. Langmuir 12, 1697-1700.

54. Weier, H.U.G. (2001) Quantitative DNA fiber mapping. Methods in Cell Biology, Vol. 64, Part B, Cytometry, Third Edition (Z. Darzynkiewicz, H.A. Chrissman and J.P. Robinson, Eds.) Academic Press, San Diego, pp.37-56.

55. Weier, H.-U.G. (2001) DNA fiber mapping techniques for the assembly of high-resolution physical maps. J. Histochem. Cytochem. 49, 939-948.

56. Duell, T., M. Wang, J. Wu, U.-J. Kim, H.-U.G. Weier (1997) High resolution physical map of the immunoglobulin lambda variant gene cluster assembled by quantitative DNA fiber mapping. Genomics 45, 479-486.

57. Duell, T., Nielsen, L.B., Jones, A., Young, S.G., Weier, H.-U. G. (1998) Construction of two near-kilobase resolution restriction maps of the 5´ regulatory region of the

human apolipoprotein B gene by quantitative DNA fiber mapping (QDFM). Cytogenet. Cell. Genet. 79, 64-70

58. Breier, A.M., Weier, H.-U.G., and Cozzarelli, N.R. (2005) Independence of replisomes in *Escherichia coli* chromosomal replication. Proc. Natl. Acad. Science USA 102, 3942-3947.

59. Weier, H.-U.G. (2002) Quantitative DNA fiber mapping, in *FISH Technology* (Rautenstrauss, B. and Liehr , T. ,eds.), Springer Verlag, Heidelberg, FRG, pp.226-253.

60. Hsieh, H.B., M. Wang, R. A. Lersch, U.-J. Kim, and H.-U. G. Weier (2000) Rational Design of Landmark Probes for Quantitative DNA Fiber Mapping (QDFM). Nucl. Acids Res. 28, e30.

61. Cassel, M.J., Munné, S., Fung, J., and Weier, H.-U.G. (1997) Carrier-specific breakpoint-spanning DNA probes for pre-implantation genetic diagnosis [PGD] in interphase cells. Hum. Reprod. 12,101-109.

62. Fung, J.L., Munné S., Garcia J., Kim U.-J., Weier H.-U.G. (1999) Reciprocal translocations and infertility: molecular cloning of breakpoints in a case of constitutional transcolation t(11;22)(q23;q11) and preparation of probes for preimplantation genetic diagnosis (PGD). Reprod., Fertility and Devel. 11, 17-23.

63. Collins, C., Rommens, J.M., Kowbel, D., Godfrey, T., Tanner, M., Hwang, S.I., Polikoff, D., Nonet, G., Cochran, J., Myambo, K., Jay, K.E., Froula, J., Cloutier, T., Kuo, W.L., Yaswen, P., Dairkee, S., Giovanola, J., Hutchinson, G.B., Isola, J., Kallioniemi, O.P., Palazzolo, M., Martin, C., Ericsson, C., Pinkel, D., Albertson, D., Li, W.B., and Gray JW. (1998) Positional cloning of ZNF217 and NABC1: genes amplified at 20q13.2 and overexpressed in breast carcinoma. Proc. Natl. Acad. Sci. U.S.A. 95, 8703-8708.

Table I. Mapping ~3 kb sequencing templates on DNA fibers prepared from P1 #39.

clone ID	proximal position (kb)	distal position (kb)	plasmid insert size (kb)[1]	homology with vector	chimeric clone	vector map position
1-h3	80 +/- 1.6	83 +/- 1.3	2.6	No	No	-
9-d4	77.3 +/- 1.4	79.6 +/- 1.3	2.9	No	No	-
2-a2	76.3 +/- 1.3	79.5 +/- 1.3	3.2	No	No	-
4-c11	74 +/- 2.2	77.5 +/- 1.9	3.4	No	No	-
10-h8	55.1 +/- 1.7	58.8 +/- 1.9	3.6	No	No	-
4-h3	54.3 +/- 3.2	58.8 +/- 3.2	4.5	No	No	-
4-h5	52.3 +/- 3.8	56.4 +/- 4.2	6.4 {2.3, 4.1}	Yes	Yes	4500-7500
1-c7 signal1	0	3.4 +/- 0.6	8.0	Yes	Yes	Sal 1 side
1-c7 signal2	82.8 +/- 4.1	85.1 +/- 4.2		No		Not 1 side

[1]Measured by agarose gel electrophoresis.

Figure Legends

Fig. 1. Strategies for assembly of high resolution physical maps. (**A**) STS's are generated along the YAC clone (solid black line) and used to screen P1, PAC or BAC libraries by PCR. This allows to order the clones and group them into contigs. (**B-D**) Physical map assembly using QDFM. (**B**) Mapping of small clones such as clones A and B shown here onto larger DNA molecules (i.e., BACs, PACs, YACs) allows measurement of clone size (’size 2’), distance of the hybridization domains from either end of the larger molecule (’a ’, ’b ’, ’c ’) and between clones ('gap'). (**C**) Mapping of one clone relative to another allows determination of overlap between clones and their relative orientation. Hatched boxes indicate vector DNA-specific sequences that can be tagged with specific DNA probes.

Fig. 2. Orientation and stretching of DNA molecules using the surface tension of a receeding meniscus. (**A-C**) Schematic diagram showing DNA molecules bound at one or both ends, and being pulled in the direction of the receeding menicus during drying. (**D-F**) High density of lambda DNA molecules after ’molecular combing’ and hybridization with DNA probes specific for individual Hind III fragments. These gray-scale images show either the red or green signals observed after detection of bio-tinylated or digoxigenin(dig.)-labeled probes (F,E) or the superposition of both (D).

Fig. 3. Application of QDFM for the precise localization of a genomic interval represented by a P1 clone along a larger YAC DNA molecule. (**A-C**) This dual color FISH experiment allows to measure the physical distances from the ends of the YAC (distances A,B) as well as the overlap between these clones (’overlap’). The image

in (A) shows the superposition of red (B) and green (C) images recorded from dig.-labeled and biotinylated probes, respectively.

Fig. 4. Physical mapping of P1 clones and estimation of the size of a gap. **(A)** The P1 clones 1143 and 1107 represent the ends of two contigs of P1 clones that map to the long arm of chromosome 21. The YAC clone 141G6 carries an insert of about 475 kb that contains the same genomic region. **(B)** Hybridization of two dig.-labeled P1 probes (detected with anti-dig-rhodamine, red) and a biotinylated DNA probe prepared from YAC DNA (detected with avidibn-FITC, green) allows the measurement of the positions of the P1 probes in the larger genomic interval defined by the YAC insert. The same QDFM experiment also provides an accurate measurement of the distance between the two P1 clones (’gap’). **(C)** The extent of the gap between clones '1143' and '1107', was determined to be 10.9 μm or 25.4 kb (±1.1 kb) by measuring the physical distances between the P1 hybridization signals on 10 YAC fibers.

Fig. 5. High resolution mapping of sequencing templates (~3 kb plasmid clones) along a human P1 clone. Six plasmid clones were hybridized onto DNA fibers prepared from Not 1-digested DNA of clone P1 #39. The position of the plasmid-specific hybridi-zation signals is indicated by arrows. Two signals, in the insert (arrow) and in the vector part (arrow head), were detected when hybridizing a probe prepared from plasmid clone 4-h5. The horizontal bar indicates the part of the DNA fiber representing the P1 vector.

Fig. 6. High resolution mapping of contigs allows selection of non-overlapping clones for parallel sequencing. **(A)** Mapping on a contig comprised of 5 plasmid clones onto linearized P1 #30 fibers. **(B)** The absolute positions of 3 plasmid contigs along P1 #30. The numbers indicate mean values of distances or contig sizes in kb.