

tion of particles, such as polystyrene particles, which compete with DNA for binding to ethidium bromide. With this technique, the filament can be densely labeled during the filament extension operation, to permit easy visualization of the extended molecule. Thereafter, for examining any reactions of molecules with the filament, the staining dye can be removed so that the dye will not interfere with these reactions. Also removal of the dye may be necessary for contrast enhancement, in order to visualize fluorescent-labeled molecules bound to the extended filament.

Alternatively, the binding molecule can be labeled with a reporter having a different fluorescence absorption peak, allowing the second reporter to be visualized at a second excitation wavelength. Fluorescent-labeled probes suitable for labeling probes, enzymes and or particles are well known. In one embodiment, for use in detecting single-reporter fluorescent events, the illumination source is preferably a pulsed laser which can be operated at high power levels over timed pulsed intervals as short as 10^{-12} to 10^{-9} seconds. As discussed above, the fluorescence from the reporter is observed only in the interval between excitation pulses, to eliminate background Raman scattering.

For high-resolution, i.e., resolution at the level of a few basepairs, the extended DNA filament can be examined by nanometer-scale probe microscopy, scanning tunnelling microscopy (e.g., Dunlap, Williams), or dehydrated and examined by conventional or scanning electron microscopy.

FIGS. 9A and 9B illustrate a method for examining extended nucleic acid filaments on a substrate in a dehydrated form. Here a nucleic acid filament **130** is extended and fixed in the liquid film in the chamber, as above, over a substrate **132** in the chamber, indicated at **134** in FIG. 9A. The filament in solution may be contacted with a selected binding molecule, such as sequence-specific oligonucleotide probes, binding proteins, enzymes, histone proteins, ribosomal particles or the like, as described in Section III below, to bind the agent at a site on the filament. The chamber is then drained and the filament is allowed to dry, in its extended form, on the substrate, as shown in top view in FIG. 9B. For examination by transmission electron microscopy, the filament can be stained with conventional tungstate salts or the like. For examination by scanning electron microscopy or force field microscopy, the filament may be metalized, or examined directly.

The advantages of the polymer manipulation method of the invention can be appreciated from the foregoing. The method facilitates particle manipulation by maintaining a relatively constant trapping force on the particle as the particle is moved in the view field. In particular, the particle can be manipulated within the view field at a selected trapping force, and extended to a length corresponding to a known, selected stretching force. This, in turn, provides a standard measure of polymer length, in the extended-filament condition, which can be calibrated in terms of number of polymer subunits.

The method also provides a simple method for attaching the ends of a stretched filament to the chamber, using the optical trap to adhere the particles at the filament ends to the chamber.

According to another feature, the method can be used to extend extremely large nucleic acid fragments, such as genomic fragments in the 1-10 megabasepair

size range or larger. Fragments of this size are quite fragile and previous methods for physically extending the fragments have generally been unsuccessful, due to the inability to control the stretching force applied to the filament. In the present method, the stretching force exerted on the filament is never greater than the trapping force exerted on the filament-coupled particle, and this force can be selected to ensure that the filament is not broken as it is extended.

III. Nucleic Acid Filament Preparation

In another aspect, the invention includes a method of nucleic acid filament preparation, for examining the filament in an extended condition. In one general embodiment, the filament is contacted with a sequence-dependent binding molecule, and the binding site(s) in the extended filament are localized by determining the distance from a site from the ends of the filaments, or from one another.

This method is illustrated by the probe localization method described below with respect to FIGS. 10A and 10B, which illustrate a method for restriction-fragment mapping of an entire genomic chromosomal DNA filament. The filament, indicated at **140** in FIG. 11A, is a 1-10 megabasepair genomic duplex fragment having rare restriction sites S_n spaced at intervals having an average spacing, for example of 100-1,000 kilobases. Examples of rare restriction sites are XhoI, with an average spacing between sites of about 200 kbases, SfiI and MluI, with an average spacing of about 500 kbases, and NotI, with an average spacing of about 1,000 kilobases.

The genomic fragments are prepared according to known methods. Where, as here, it is desired to extend an entire chromosomal DNA, isolation must be done with a minimum of disruptive handling procedures. In one known method, chromosomal DNA can be isolated from a cell by treating the cell with proteases and cell disruptive agents to release the chromosomal DNA, which is then drawn into an agarose slab and fractionated by agarose electrophoresis. The selected fragment may be eluted by electrophoresis into a receiving chamber which becomes the viewing chamber where particle attachment to the filament(s) and particle manipulation are carried out.

The genomic filaments are suspended in a standard coupling buffer and the fragment ends are coupled to amine-coated beads, such as beads **142**, **144** coupled to fragment **140**. The buffer is then replaced by a standard hybridization buffer containing 1% by weight methylcellulose (50-100 kdaltons), at a fragment concentration of about 10^9 filaments/cc, as above.

To the fragment mixture is added a fluorescent-labeled probe, such as DNA probe **146**, which is complementary to the selected rare restriction site sequences, such as the NotI sites in the fragments. The probes are mixed with the duplex fragments under partial denaturation conditions which allow probe hybridization with the duplex fragment, according to known methods. Alternatively, the probes may be hybridized to the duplex by RecA-catalyzed D-loop formation. Fluorescent-labeled probes are prepared conventionally.

Where it is desired to examine a fragment containing a known sequence, such as sequence A in FIG. 10A, the desired fragment may be identified by its binding to a fluorescently-labeled probe **148** specific to the known region, but distinguishable from the restriction-site