Fluorescent labeling of nucleic acids with high density is very important for a wide area of DNA- and RNA-based diagnostics [1–5], but it still remains a challenge. Indeed, chemical synthesis of oligonucleotides with multiple fluorophores is an elaborate and expensive procedure [6–8]. Branched probes tagged with numerous fluorophores can alleviate this problem [2,9], but special complex assemblies are required in that case. Enzymatic synthesis of high-density labeled polynucleotides from fluorescently modified monomers by DNA polymerases is another alternative. However, despite some progress in this area, there are certain difficulties in enzymatically generating long fluorescent products using procedures based on primer extension [3,4,10–13].

Rolling-circle amplification (RCA), which is isothermal DNA polymerase-driven cyclic replication of small circular DNA probes, is often used as an efficient method to detect various analytes [14–16]. It was assumed that the RCA mechanism is different from that of primer-extension reactions using linear DNA templates. Based on that, we suggested that DNA polymerase during RCA might better tolerate fluorescently labeled deoxynucleotide triphosphates (dNTPs) and decided to investigate whether RCA can be employed for direct generation of densely labeled amplification products capable of intense fluorescence.

We chose Φ29 DNA polymerase considering its well-known robust RCA activity [23–25] and the hydrophilic dye Cy3 with which high-density labeling could be achieved without serious fluorescence quenching [8,26]. All experiments were performed with Cy3-dCTP and Cy3-dUTP, which substituted dCTP and dTTP, respectively, and with 88-nt DNA minicircle carrying Salmonella sequence marker [27].

Gel-electrophoretic analysis of RCA products obtained in solution with complete substitution of two pyrimidine dNTPs with Cy3-tagged fluorescent analogs showed that the efficiency of synthesis was reduced as compared to that with non-modified dNTPs. Nevertheless,
the length of RCA amplicons reached 1 to 2 kb (10–20 repeats) as compared with size standards (data not shown). Keeping in mind that multiply labeled DNA exhibits altered physicochemical properties, including anomalous gel-electrophoretic mobility [10,12], we decided to directly measure the length of RCA products using electron microscopy (EM). EM visualization of Cy3-labeled RCA products (Fig. 1A) showed that the length of randomly chosen amplicons ranged from 400 bp to 3 kb (Fig. 1B), with the average size of 1.2 kb being in agreement with initial gel-electrophoretic estimations.

Thus, these results mean that every RCA amplicon carries several hundred fluorophores, and the question is whether fluorescent signal is strong enough to be detected using fluorescence imaging instruments. To answer this question, we chose the surface-immobilized RCA format, shown schematically in Fig. 2A, that uses biotin–streptavidin interactions for primer immobilization. Based on the published data [23,29,30], we expected that RCA efficiency in heterogeneous systems would be close to that in a homogeneous solution.

Using conventional fluorescent microscopy, we detected RCA amplicons as the luminous dot-like objects, the number of which correlated with the amount of primers immobilized onto a surface but was substantially less than expected (Fig. 2B). We reasoned that when using fluorescent microscopy, we probably see only the brightest amplicons while the less intense and blurred signals are lost for analysis. To improve sensitivity and make assay applicable for automation, we employed the fluorescence imaging scanner, allowing the measurement of total fluorescence of RCA amplicons per specified area (Fig. 2C).

Using this approach, we detected as few as $10^2$ amplicons/mm$^2$ (Fig. 2D), corresponding to the detection limit of approximately thousand analyte molecules.

Thus, our study shows that direct incorporation of fluorescently labeled dNTPs during RCA allows synthesis of densely labeled RCA amplicons, which can be detected with high sensitivity using fluorescent scanning instruments. Although fluorescently modified dNTPs decrease considerably the degree of RCA amplification, high-density labeling compensates for that and the assay still allows detecting down to $10^3$ molecules of the analyte. With normal dNTPs, a much higher, usually more than $10^3$-fold, amplification can be achieved [24]. However, with postreplicative procedures it is difficult to reach high-density labeling. Indeed, our studies of RCA reactions with normal dNTPs and postreplicative labeling of amplicons using dual-labeled hybridization probes demonstrated somewhat better, but comparable, sensitivity (Fig. 2D). When considering advantages of the proposed approach, we should note that the surface-immobilized format with direct high-density fluorescent labeling is well suited to high-throughput parallel analysis on diagnostic arrays. If single-molecule sensitivity...
is not an issue, direct RCA labeling and automated scanning of the arrays could be an assay of choice.

In summary, we have reported that RCA with Φ29 DNA polymerase and Cy3-labeled dNTPs is a simple tool for generation of high-density fluorescently labeled amplicons. When biotinylated RCA primer is immobilized on streptavidin-covered glass slides, the assay employing fluorescent scanners can be used for direct and sensitive quantitation of corresponding analytes without any hybridization probes.

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