Massively parallel sequencing

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I) **Pyrosequencing**

A procedure

A form of "Sequencing by synthesis".

A primer base pairs to one strand of DNA.

A DNA polymerase is used to synthesize the complementary strand.

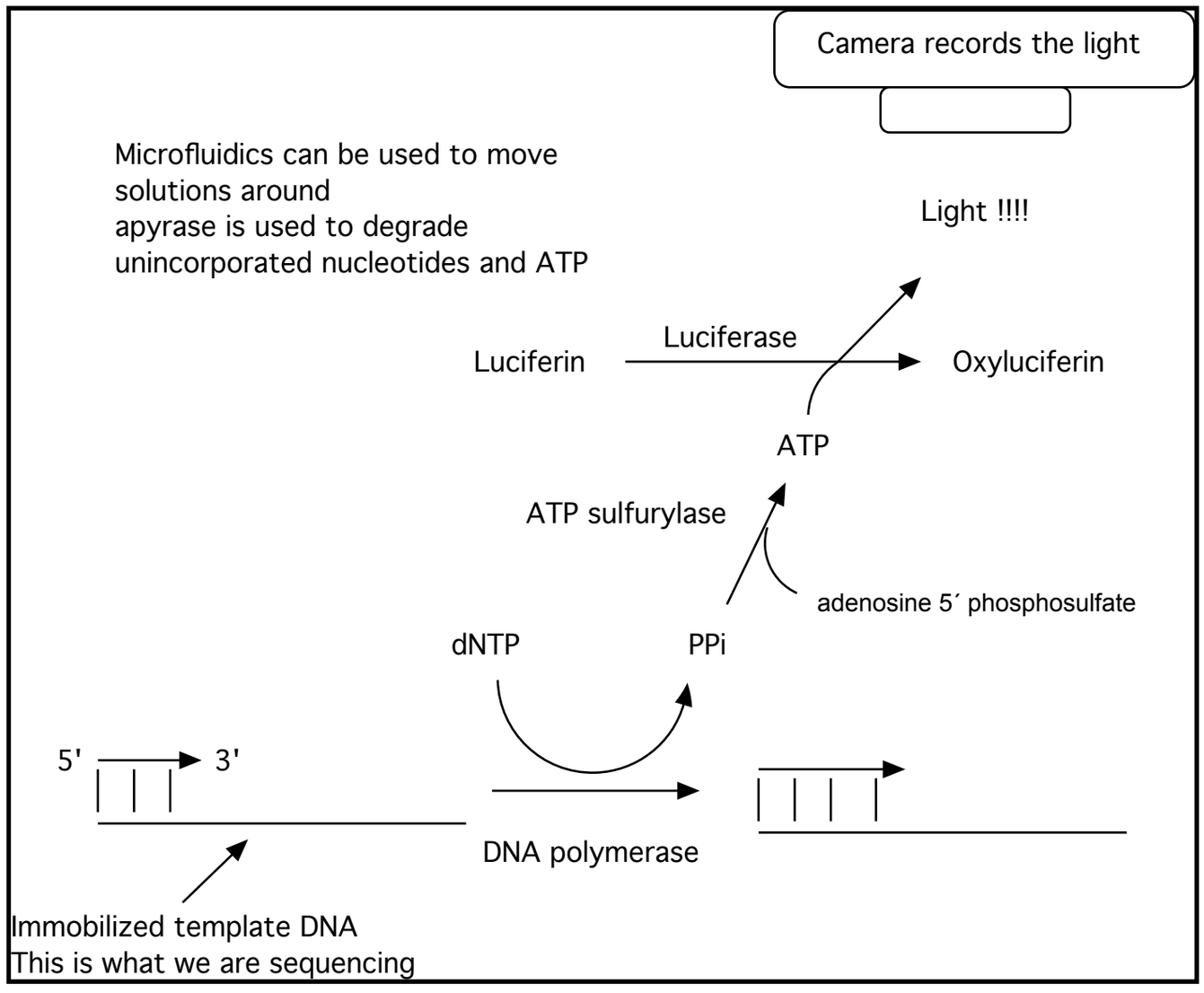
Nucleotides are added one at a time, given time to react and then washed away.

Regular dCTP, dGTP and dTTP are used by dATPαS is used in place of dATP reason given below.

3' hydroxyls of the nucleotides are blocked

Microfluidics are used to move solutions in and out.

Enzymes added are DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates adenosine 5´ phosphosulfate (APS) and luciferin.



I) **A quote about pyrosequencing from the book cited below.**

"Sequencing by synthesis" involves taking a single strand of the DNA to be sequenced and then synthesizing its complementary strand enzymatically. The Pyrosequencing method is based on detecting the activity of DNA polymerase (a DNA synthesizing enzyme) with another chemiluminescent enzyme. Essentially, the method allows sequencing of a single strand of DNA by synthesizing the complementary strand along it, one base pair at a time, and detecting which base was actually added at each step. The template DNA is immobilized, and solutions of A, C, G, and T nucleotides are added and removed after the reaction, sequentially. Light is produced only when the nucleotide solution complements the first unpaired base of the template. The sequence of solutions which produce chemiluminescent signals allows the determination of the sequence of the template.

ssDNA template is hybridized to a sequencing primer and incubated with the enzymes DNA polymerase, ATP sulfurylase, luciferase and apyrase, and with the substrates adenosine 5´ phosphosulfate (APS) and luciferin.

1. The addition of one of the four deoxynucleotide triphosphates (dNTPs)(in the case of dATP we add dATPαS which is not a substrate for a luciferase) initiates the second step. DNA polymerase incorporates the correct, complementary dNTPs onto the template. This incorporation releases pyrophosphate (PPi) stoichiometrically.

2. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5´ phosphosulfate. This ATP acts as fuel to the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a camera and analyzed in a program.

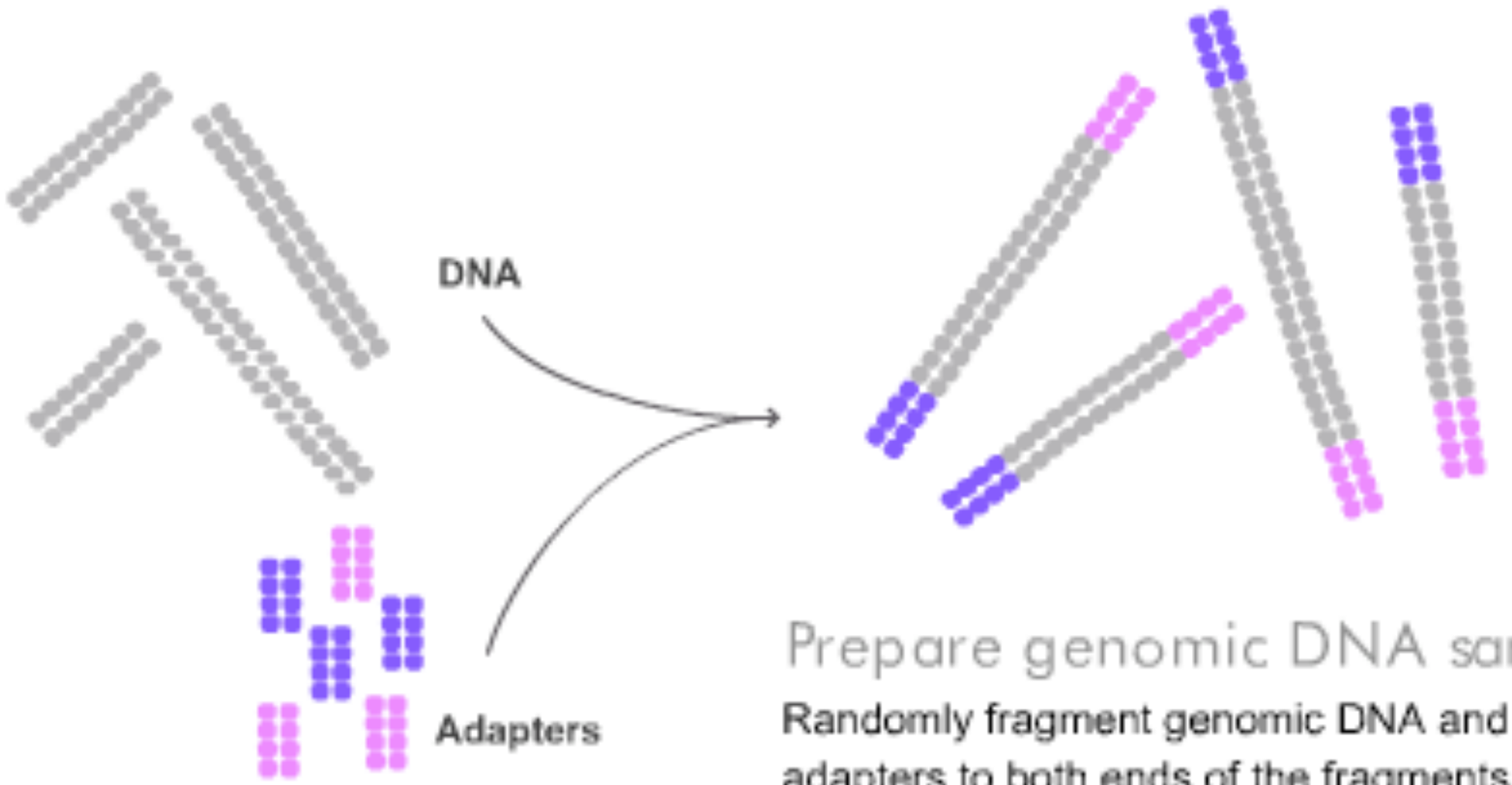
3. Unincorporated nucleotides and ATP are degraded by the apyrase, and the reaction can restart with another nucleotide.

Currently, a limitation of the method is that the lengths of individual reads of DNA sequence are in the neighborhood of 300-500 nucleotides, shorter than the 800-1000 obtainable with chain termination methods (e.g. Sanger sequencing). This can make the process of genome assembly more difficult, particularly for sequence containing a large amount of repetitive DNA. As of 2007, pyrosequencing is most commonly used for resequencing or sequencing of genomes for which the sequence of a close relative is already available.

The templates for pyrosequencing can be made both by solid phase template preparation (Streptavidin coated magnetic beads) and enzymatic template preparation (Apyrase+Exonuclease)."

1) DNA Methylation book from Doefler.

1



Prepare genomic DNA sample

Randomly fragment genomic DNA and ligate adapters to both ends of the fragments

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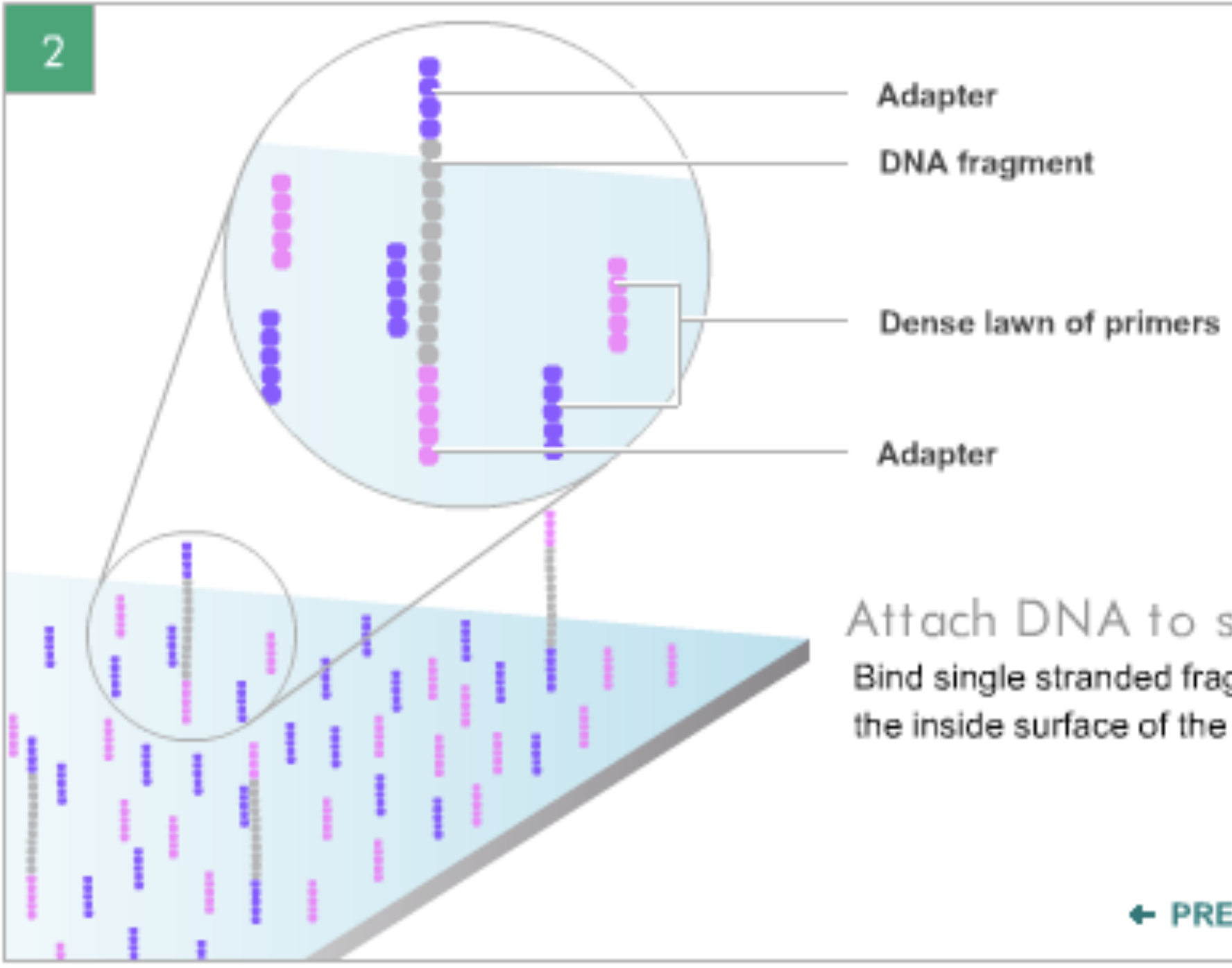

Attach DNA to surface

Bind single stranded fragments randomly to the inside surface of the flow cell channels.

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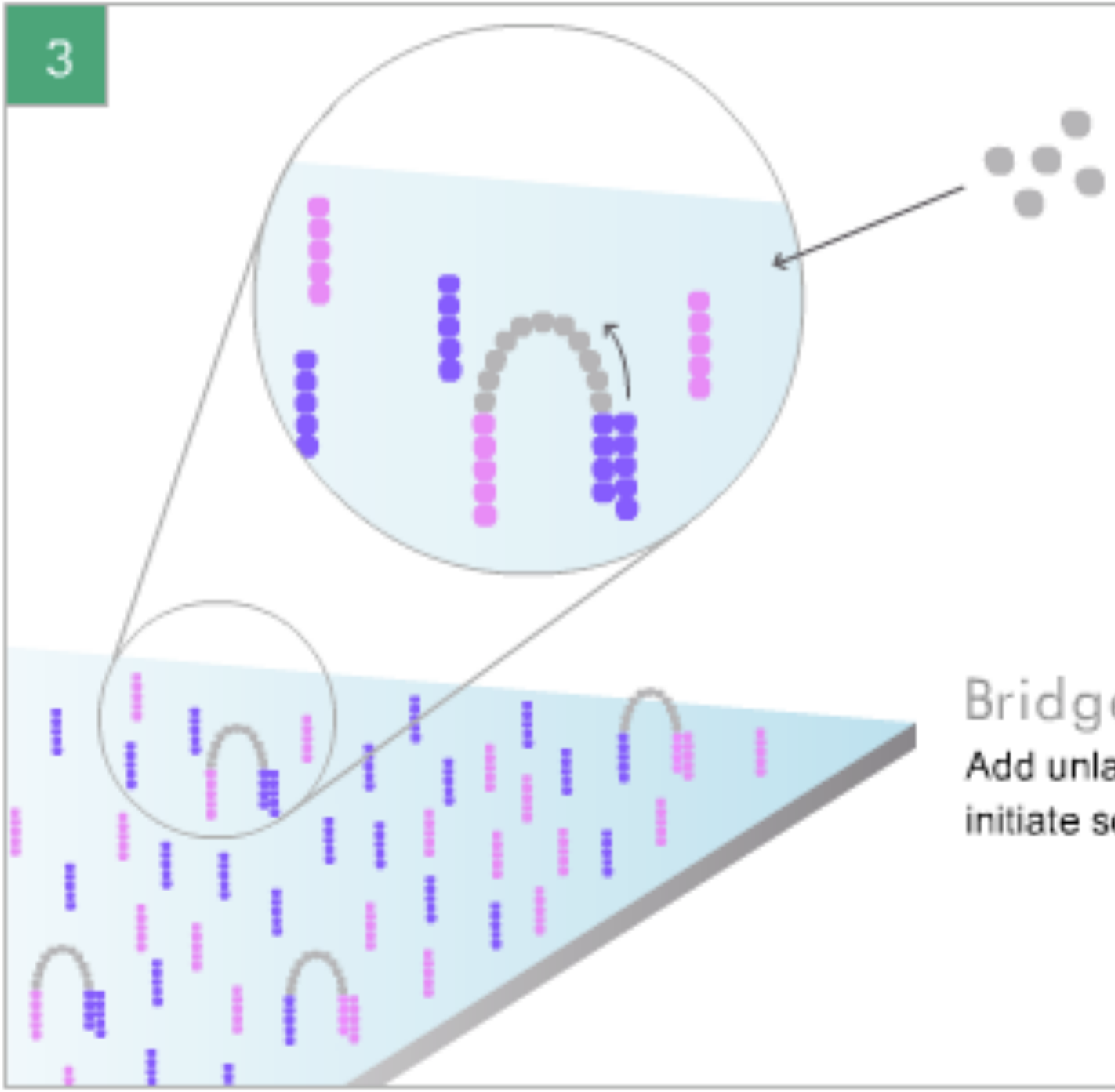

Bridge amplification

Add unlabeled nucleotides and enzyme to initiate solid-phase bridge amplification.

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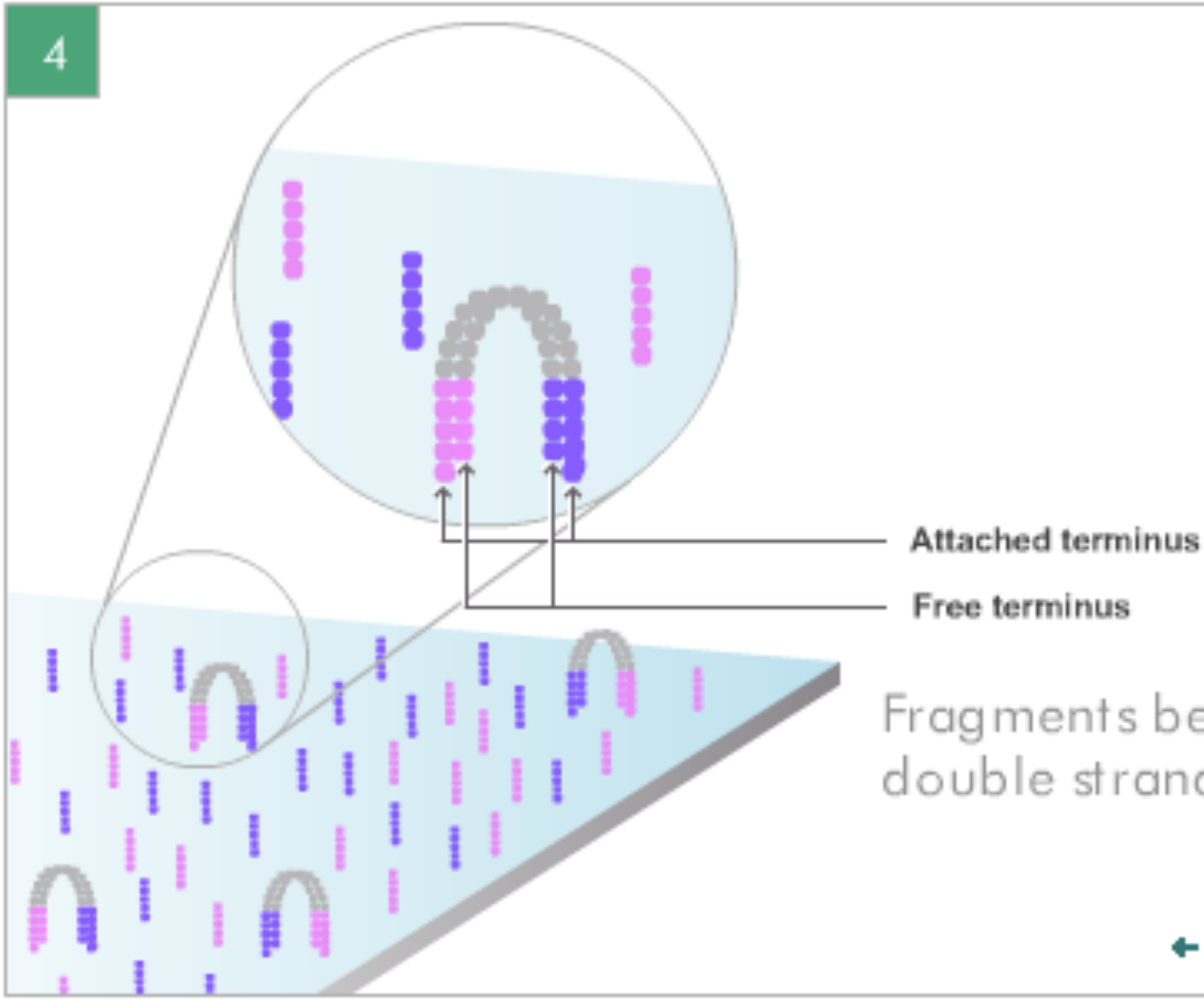

Fragments become double stranded

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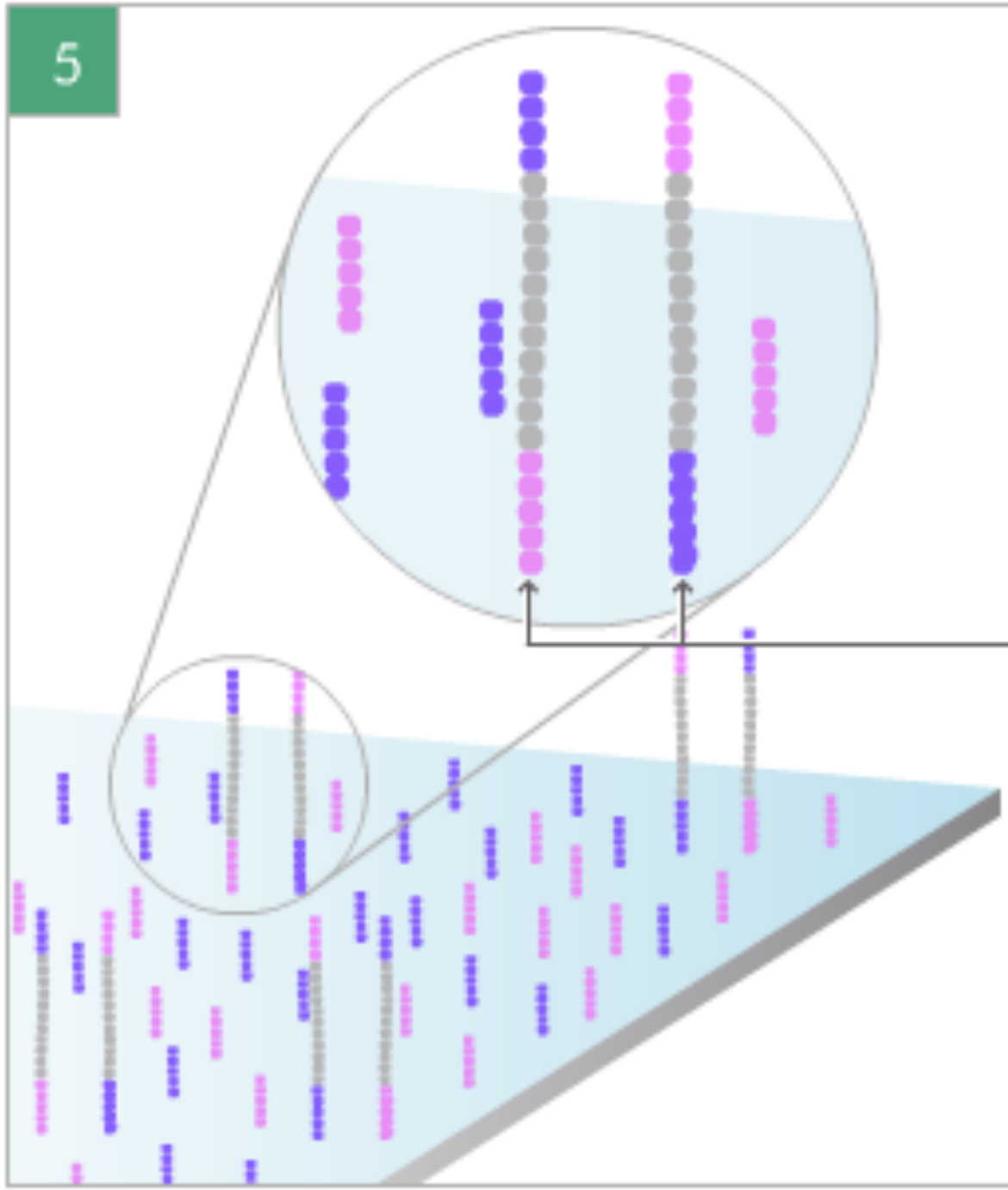

Denature the double stranded molecules

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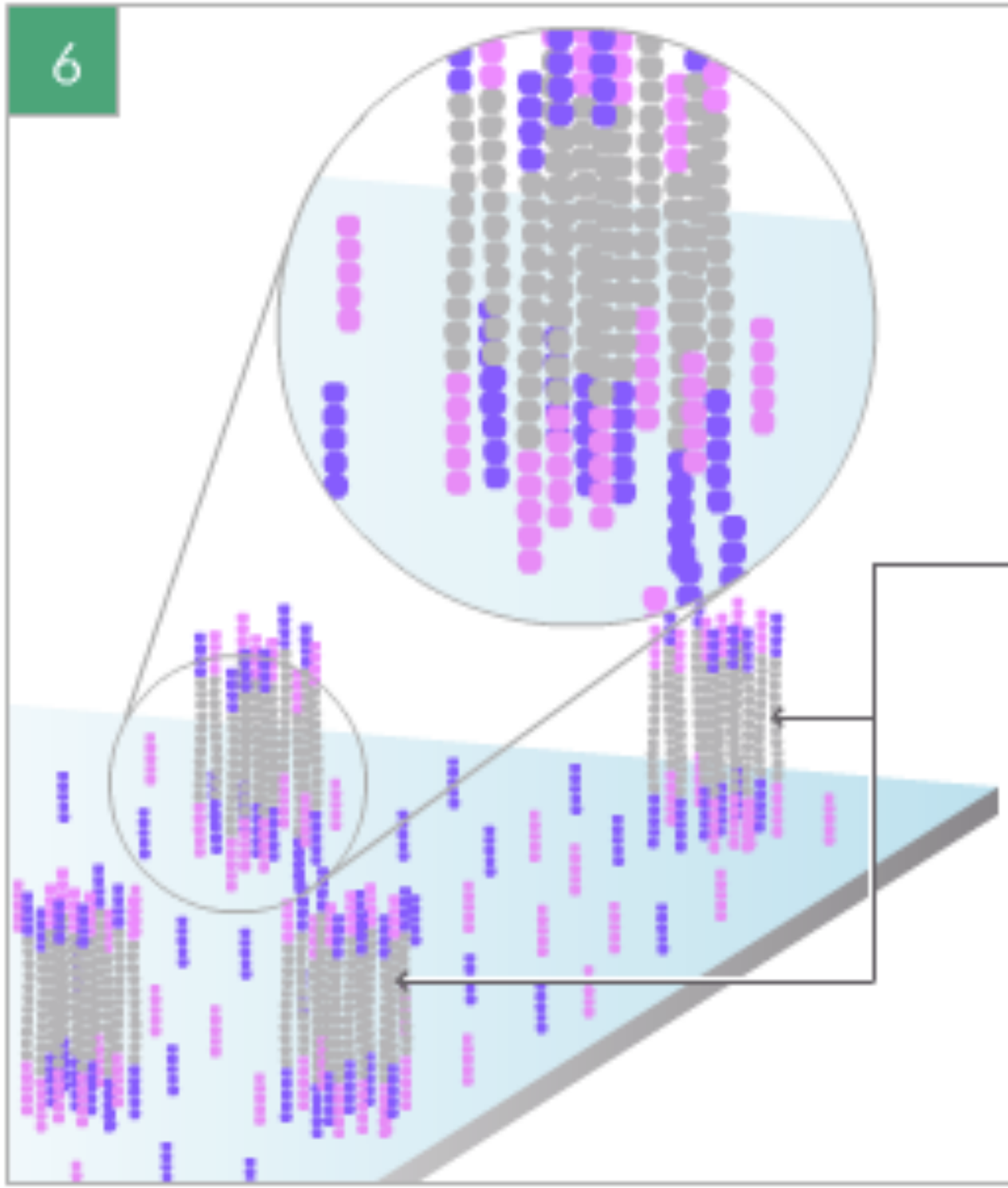

Completion of amplification

On completion, several million dense clusters of double stranded DNA are generated in each channel of the flow cell.

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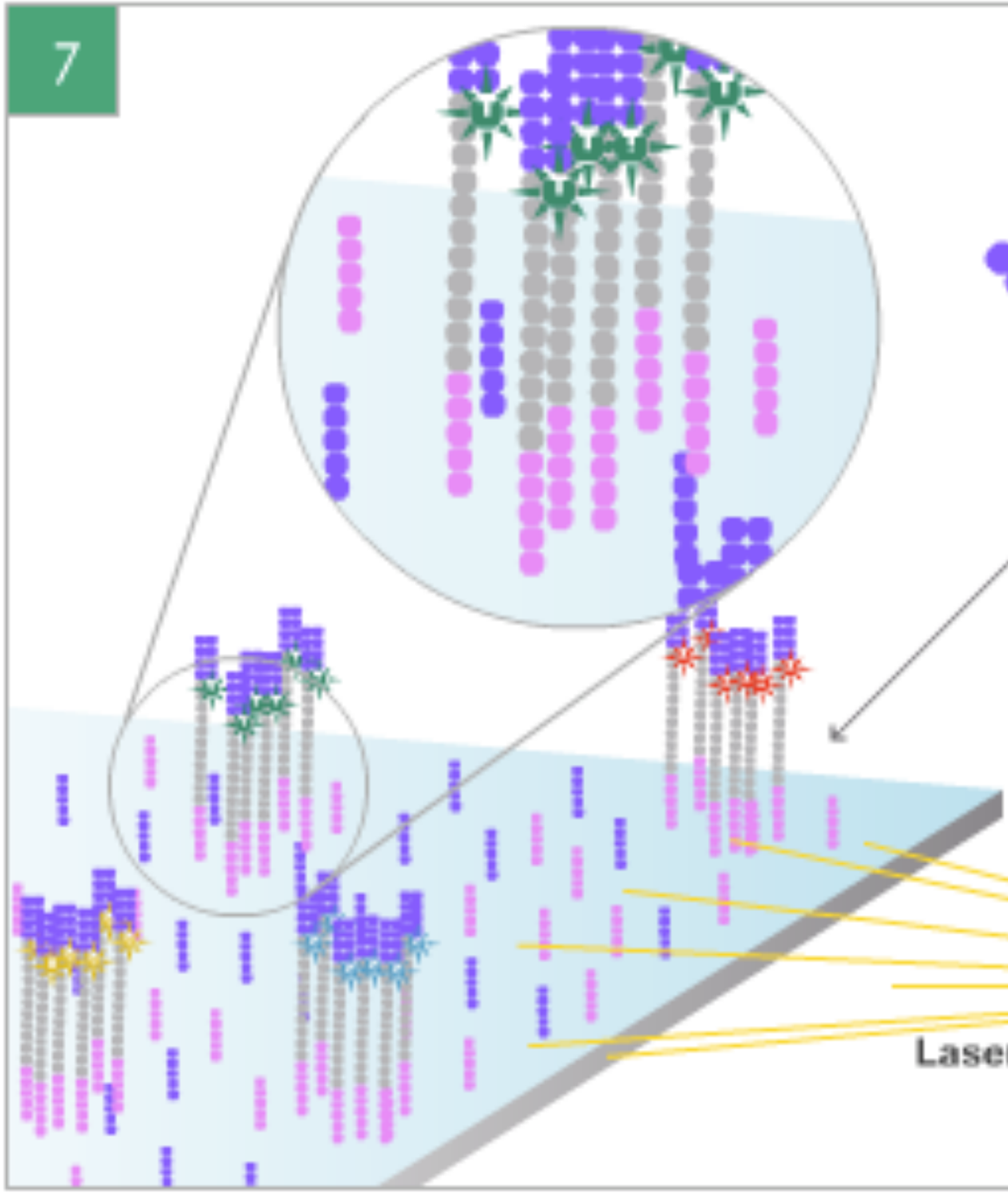

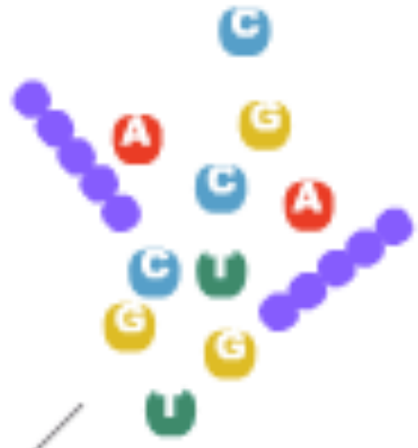

First chemistry cycle: determine first base

To initiate the first sequencing cycle, add all four labeled reversible terminators, primers and DNA polymerase enzyme to the flow cell.

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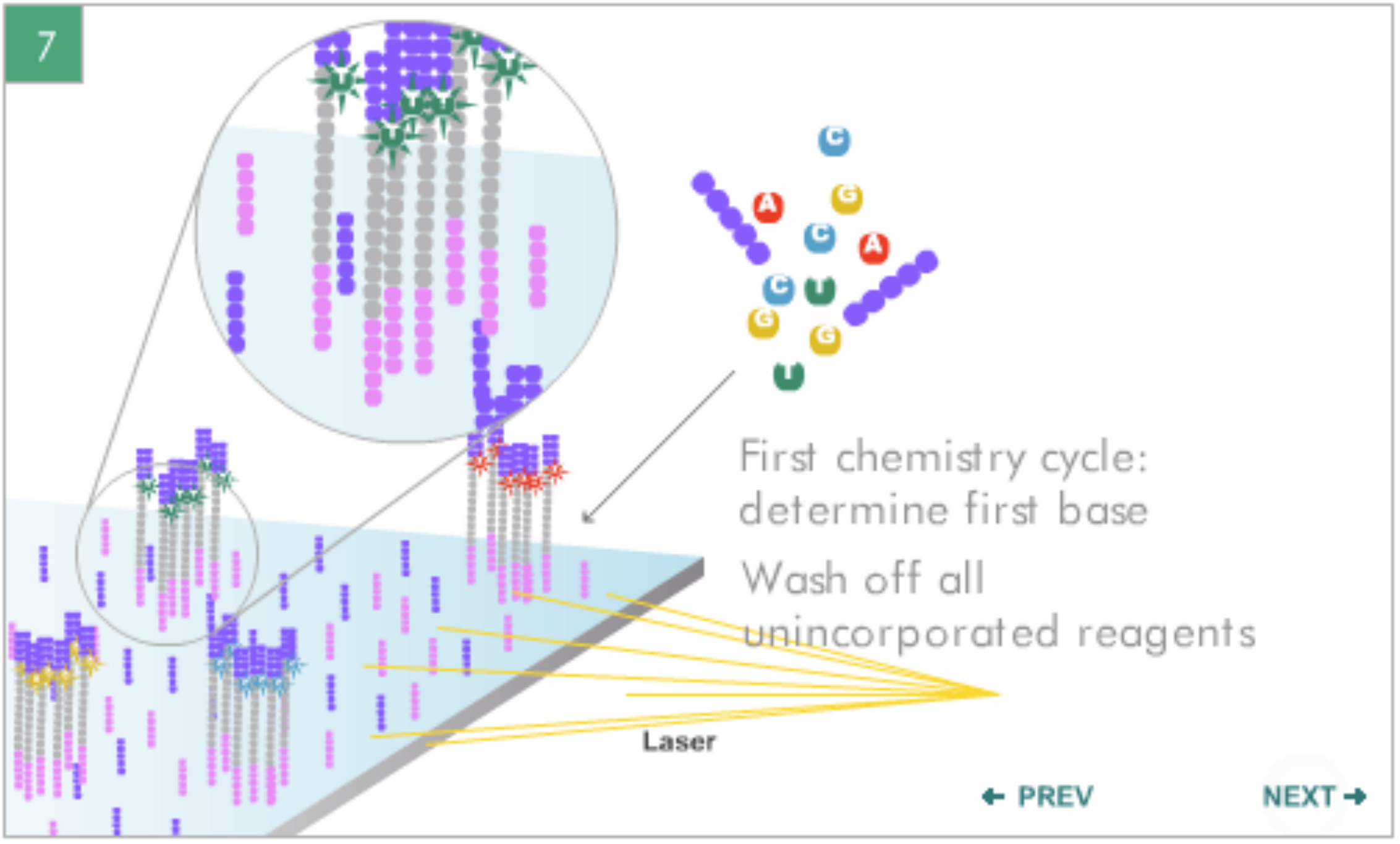
C
G
A
C
A
C
U
G
G
U
First chemistry cycle:
determine first base
Wash off all
unincorporated reagents
Laser

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Image of first chemistry cycle

After laser excitation, capture the image of emitted fluorescence from each cluster on the flow cell. Record the identity of the first base for each cluster.

Before initiating the next chemistry cycle

The blocked 3' terminus and the fluorophore from each incorporated base are removed.

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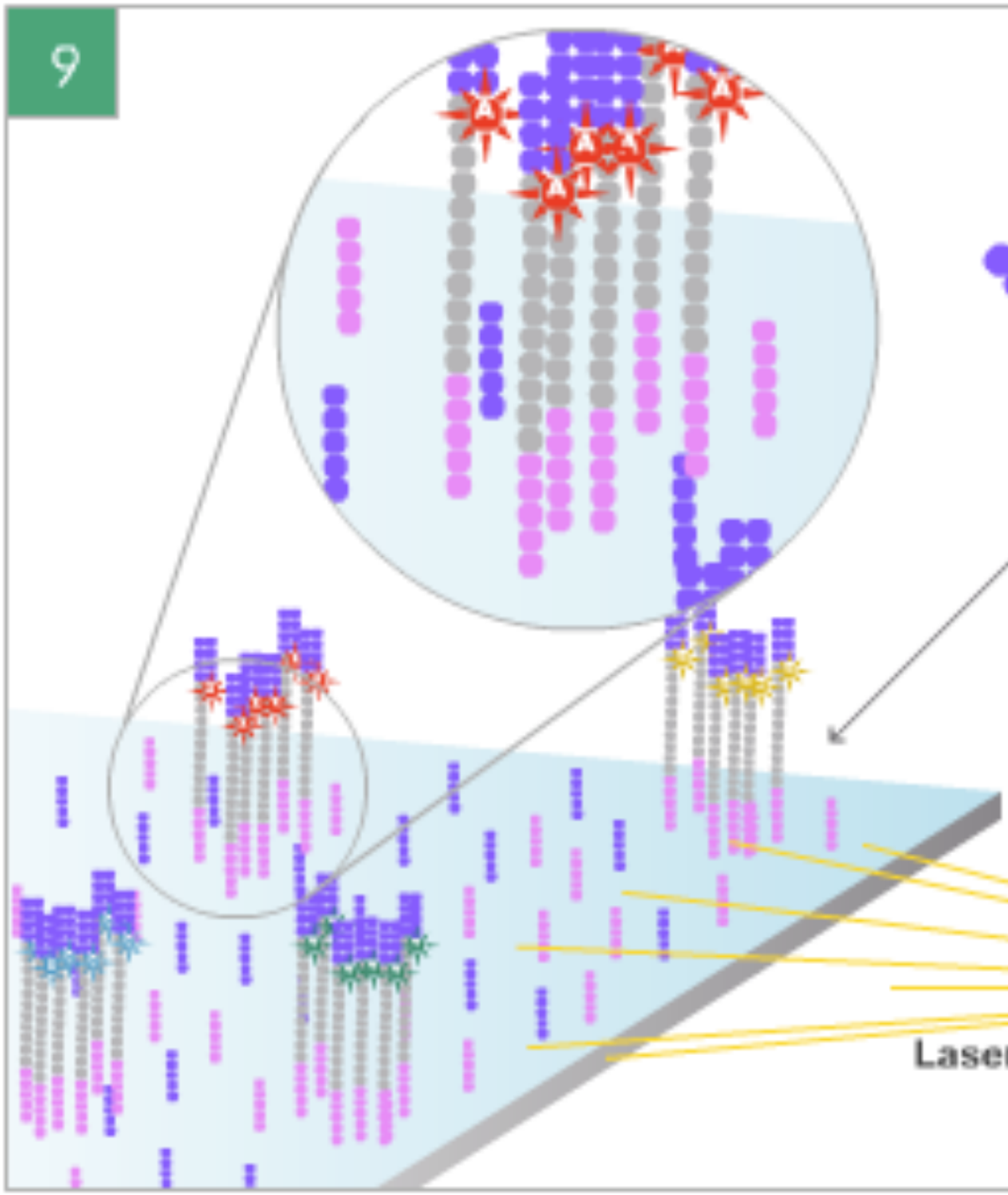

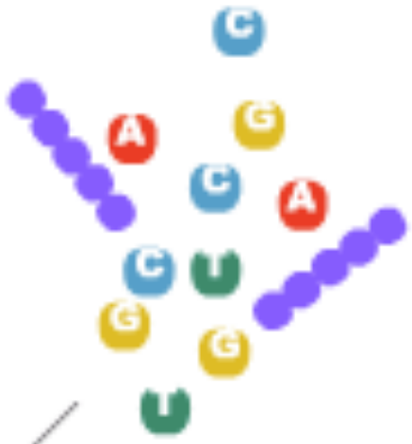

Second Chemistry Cycle: determine second base

To initiate the next sequencing cycle, add all four labeled reversible terminators and enzyme to the flow cell.

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Image of second chemistry cycle is captured by the instrument

After laser excitation, collect the image data as before. Record the identity of the second base for each cluster.

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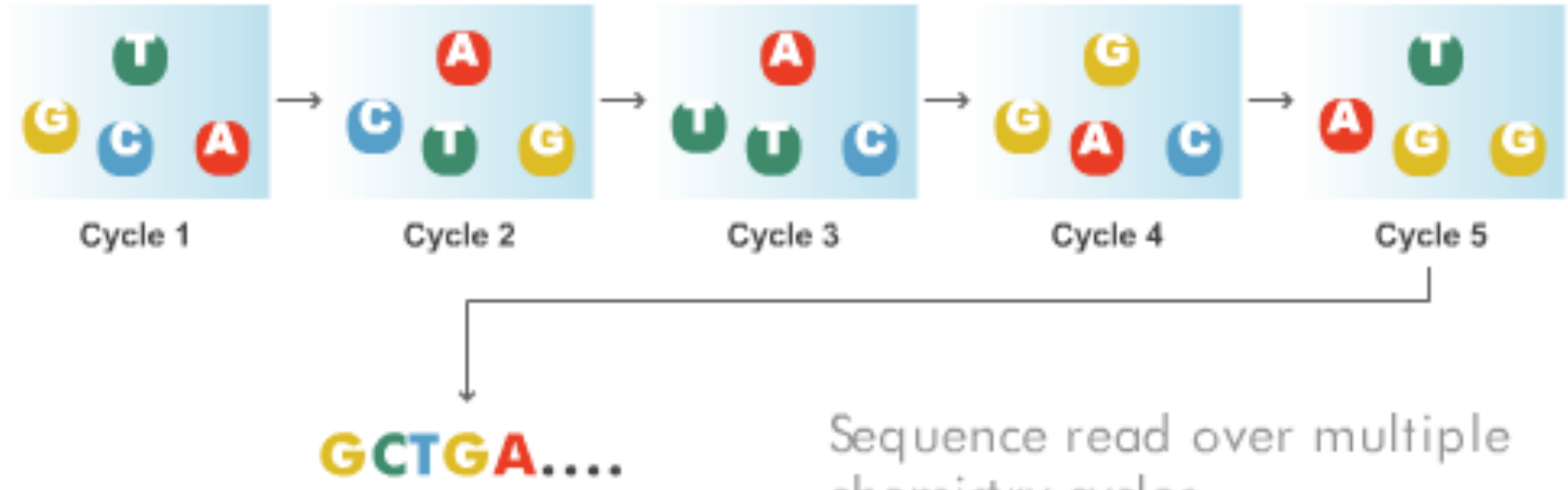

Sequence read over multiple chemistry cycles

Repeat cycles of sequencing to determine the sequence of bases in a given fragment a single base at a time.

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