

CLAIMS

We claim:

1. A method for sequencing polynucleotides comprising:

attaching a ~~single first~~ DNA template molecule to ~~each a~~ first attachment element of a plurality of attachment elements ~~on of~~ a sample container and attaching a second DNA template molecule to a second attachment element of the plurality of attachment elements, wherein the ~~average distance between adjacent elements~~ the first attachment element and the second attachment element is less than Abbe's limit;

~~applying providing~~ a stochastic photo-switching chemistry to ~~all of the~~ sample container, the stochastic photo-switching chemistry comprising a set of molecules at the same time to cause the attached molecules to fluoresce in on and off events in up to four different colors by stochastic photo-switching that have a 5' diphosphate quencher molecule and a 3' phosphate block with a label moiety, a polymerase to incorporate a nucleotide of the set of nucleotides and cleave the 5' diphosphate quencher molecule, and an enzyme to cleave the 3' phosphate block with the label moiety; and

imaging a series of on and off events for the first DNA template molecule in real-time as the on and off events are occurring, each on event occurring when the polymerase incorporates a nucleotide of the set of nucleotides and cleaves the 5' diphosphate quencher molecule and each off event occurring when the enzymes cleaves the 3' phosphate block with the label moiety; and

imaging a series of the on and off events in a color channel for each color in real-time ~~for the second DNA template in real-time~~ as the on and off events are occurring ~~for the attached molecules;~~

wherein the series of on and off events for the first DNA template molecule and the series of on and off events for the second DNA template molecule are stochastic and not synchronised.

2. The method of claim 1, wherein each of the plurality of attachment elements on the sample container is within a field of view of an imager used to image the on and off events such that imaging of the on and off events occurs at the same time for the ~~attached molecules at the plurality of attachment elements~~ first DNA template molecule and second DNA template molecule.

~~3. The method of claim 1, applying a stochastic photo-switching chemistry to all of the attached molecules at the same time comprises applying a stochastic optical reconstruction microscopy, a DNA Points Accumulation for Imaging in Nanoscale Topography, or a direct stochastic optical reconstruction microscopy stochastic photoswitching chemistry to all of the molecules at the same time.~~

~~4.3.~~ The method of claim 1, wherein due to the stochastic nature of the photo switching, the probability that an on event for a given base for a given molecule will occur at the same time as an on event for the same base at a molecule adjacent to the given molecule is less than about 0.5%.

~~5.4.~~ The method of claim 1, wherein concentrations of reagents for the stochastic photo switching are sufficient such that the probability that an on event for a given base for a

given molecule will occur at the same time as an on event for the same base at a molecule adjacent to the given molecule is less than about 0.5%.

~~6.5.~~ The method of claim 1, further comprising, controlling a rate at which the on and off events occur to control a probability that an on event for a given first nucleotide base for a given the first DNA template molecule will occur at the same time as an on event for the same base at a molecule adjacent to the given molecule nucleotide base for the second DNA template molecule based on a concentration of the first nucleotide base of the set of nucleotides and a concentration of the enzyme.

~~7.~~ The method of claim 6, wherein controlling the rate at which the on and off events occur comprises adjusting concentrations of nucleotides and enzyme in the stochastic photo-switching chemistry.

~~8.~~ The method of claim 6, wherein controlling the rate at which the on and off events occur comprises adjusting the on and off times so that the probability that an on event for a given base for a given molecule will occur at the same time as an on event for the same base at a molecule adjacent to the given molecule is lower than a determined error rate in a sequencing application in which the method is applied.

~~9.~~ The method of claim 1, further comprising, (i) determining whether an illumination intensity of a detected on event in a color channel is greater than a predetermined threshold; or

~~10.6.~~ The method of claim 1, further comprising, (ii) determining whether a spot size of a detected on event in a color channel is greater than a predetermined threshold.

~~11.7.~~ The method of claim 1, wherein the ~~average distance between adjacent elements~~
the first attachment element and the second attachment element is less than about 20 nm.

~~12.8.~~ The method of claim ~~1~~ 7, wherein the ~~average distance between adjacent elements~~
the first attachment element and the second attachment element is within a range of ~~about~~
2 nm to ~~about~~ 20 nm.

~~13.9.~~ An imaging ~~system comprising~~ assembly for preforming the method of claim 1
comprising:

a sample container comprising a plurality of attachment elements wherein ~~a single~~
the first DNA template molecule is attached to ~~each of the attachment elements~~ the first
attachment element and the second DNA template molecule is attached to the second
attachment element, and further wherein the average distance between adjacent
attachment elements is less than Abbe's limit; and

an imager positioned to image photo-switching occurring at the ~~plurality of first~~
attachment elements and the second attachment element by capturing on and off events in
a plurality of color channels at the same time as the on and off events are occurring for the
~~attached molecules~~ first DNA template molecule and the second DNA template molecule
when ~~a~~ the stochastic photo-switching chemistry is applied to ~~all of the attached molecules~~
~~at the same time causing the attached molecules to fluoresce in the on and off events in up~~
~~to four different colors.~~

~~14.10.~~ The imaging ~~system assembly~~ of claim ~~13~~ 9, wherein the sample container comprises
a flowcell that comprises the plurality of attachment elements at a plurality of sample
locations.

~~15.11.~~ The imaging ~~system assembly~~ of claim ~~13~~ 9, wherein each of the plurality of attachment elements on the sample container is within a field of view of the imager used to image the photo-switching such that the capturing of the on and off events occurs at the same time ~~for the attached molecules at the plurality of attachment elements.~~

~~16.~~ The imaging system of claim ~~13~~, the stochastic photo-switching chemistry applied to all of the attached molecules at the same time comprises a stochastic optical reconstruction microscopy, a DNA Points Accumulation for Imaging in Nanoscale Topography, or a direct stochastic optical reconstruction microscopy stochastic photoswitching chemistry.

~~17.12.~~ The imaging ~~system assembly~~ of claim ~~13~~ 9, wherein concentrations of reagents for the stochastic photo switching are sufficient such that the probability that an on event for a given base for a given molecule will occur at the same time as an on event for the same base at a molecule adjacent to the given molecule is less than 0.5%.

~~18.13.~~ The imaging ~~system assembly~~ of claim ~~13~~ 9, wherein a rate at which the on and off events occur yields a probability that an on event for a given base for a given molecule will occur at the same time as an on event for the same base at a molecule adjacent to the given molecule is lower than a determined error rate in a sequencing application in which the method is applied.

~~19.~~ The imaging system of claim ~~13~~, further comprising, determining whether an illumination intensity or a spot size of a detected on event in a color channel is greater than a predetermined threshold.

~~20.14.~~ The imaging ~~system-assembly~~ of claim ~~13~~ 9, wherein the average distance between adjacent elements is less than ~~about~~ 20 nm.

~~21.~~ A method comprising:

~~(a) providing an array of nucleotide sequences anchored to a solid support, wherein the average distance between adjacent anchors is less than Abbe's limit;~~

~~(b) providing a mixture to the array comprising an enzyme capable of coupling nucleotides, a deblocking agent, a nucleic acid bound to a strand of nucleotides having a sequence complimentary to the nucleotide sequences anchored to the solid support, and more than one nucleotide analog comprising a base with a label moiety and corresponding quencher moiety bound thereto wherein the label moieties are correlated with a specific base moiety; and~~

~~(c) allowing sequential addition of a plurality of the nucleotide analogs to the nucleic acid to proceed via several reaction cycles in the mixture while concurrently imaging the label moieties within the array;~~

~~wherein each reaction cycle comprises:~~

~~(i) the polymerase adding a nucleotide analog to the nucleic acid by cleaving the quencher moiety and forming a transient nucleic acid species comprising the label moiety; and~~

~~(ii) the deblocking agent modifying the transient nucleic acid species to remove the label moiety.~~

~~22.~~ The method of claim 21, wherein the average distance between adjacent anchors is less than about 20 nm.

~~23. The method of claim 21, wherein the average distance between adjacent anchors is within a range of about 2 nm to about 20 nm.~~

~~24. The method of claim 21, wherein the nucleotide analog comprises a pentose moiety having a 3' carbon and the label moiety is attached to the nucleotide at the 3' carbon.~~

~~25. The method of claim 21, wherein the nucleotide analog comprises a triphosphate moiety and wherein the quencher moiety is attached to the triphosphate moiety.~~

~~26. The method of claim 21, wherein the deblocking agent comprises a phosphoesterase enzyme.~~

~~27. The method of claim 26, wherein the phosphoesterase selectively removes a phosphodiester moiety or the phosphotriester moiety from the transient nucleic acid species.~~

~~28. The method of claim 26, wherein the phosphoesterase is selected from the group consisting of Endonuclease IV and AP endonuclease.~~

~~29. The method of claim 21, wherein the transient nucleic acid species is present for at least 1 millisecond before the deblocking agent modifies the transient nucleic acid species to remove the label moiety.~~

~~30. The method of claim 29, wherein the transient nucleic acid species is present for no more than 30 seconds before the deblocking agent modifies the transient nucleic acid species to remove the label moiety.~~

~~31. The method of claim 21, wherein the several reaction cycles comprises at least 100 reaction cycles, whereby the nucleic acid is extended by addition of at least 100 nucleotide analogs.~~

~~32. The method of claim 21, wherein the enzyme capable of coupling nucleotides comprises a polymerase, a myosin, or a kinase.~~