

~~What is claimed is:~~ **Claims**

1. An imaging system comprising:
an analysis substrate comprising:
a localization layer to be provided with a sample comprising a nucleotide provided with a fluorescent dye; and
a sensor layer, the localization layer being on-chip relative to the sensor layer;
and
imaging circuitry to image the sample, the imaging circuitry comprising:
an array of sensor pixels at the sensor layer, one or more of the array of sensor pixels to receive a propagation of fluorescence from the fluorescent dye; and
fitting circuitry to identify, using a signal of the sensor layer, a pixel intensity distribution due to a point spread function, and determine a centroid of the pixel intensity distribution by fitting a function to the pixel intensity distribution.
2. The imaging system of claim 1, wherein the analysis substrate is part of a flowcell.
3. The imaging system of any of claims 1 or 2, wherein the localization layer comprises nanowells.
4. The imaging system of any of claims 1 or 2, wherein the localization layer comprises a zero-mode waveguide.
5. The imaging system of any of claims 1 to 4, wherein the localization layer comprises a surface passivation layer.
6. The imaging system of any of claims 1 to 5, wherein the sensor layer comprises a complementary metal-oxide-semiconductor chip.
7. The imaging system of any of claims 1 to 6, wherein the array of sensor pixels comprises an array of photodiodes.
8. The imaging system of any of claims 1 to 7, wherein the sensor layer comprises a charge-coupled device.
9. The imaging system of any of claims 1 to 8, wherein the analysis substrate further comprises a spacer layer between the localization layer and the sensor layer, the spacer layer having a thickness based on the propagation of fluorescence from the fluorescent dye.
10. The imaging system of claim 9, wherein the spacer layer comprises a planar waveguide.
11. The imaging system of any of claims 9 to 10, wherein the thickness of the

spacer layer corresponds to the propagation of fluorescence from the fluorescent dye being received by a predefined proportion of the array of sensor pixels.

12. The imaging system of any of claims 9 to 11, wherein the thickness of the spacer layer is selected based on a predefined full width at half maximum of the propagation of fluorescence from the fluorescent dye.

13. The imaging system of any of claims 1 to 12, wherein the imaging circuitry further comprises restriction circuitry restricting the fitting circuitry to fit the function to the pixel intensity distribution only in at least one area indicated by a well distribution pattern.

14. The imaging system of claim 13, wherein the imaging circuitry further comprises rejection circuitry to reject at least one localization outside the area indicated by the well distribution pattern.

15. The imaging system of any of claims 1 to 14, wherein the sensor layer provides a single field of view of the localization layer.

16. The imaging system of any of claims 1 to 15, further comprising a filter layer between the localization layer and the sensor layer, the filter layer including at least one color filter.

17. The imaging system of any of claims 1 to 16, wherein the analysis substrate does not have a filter between the localization layer and the sensor layer, and wherein the analysis substrate does not have a waveguide between the localization layer and the sensor layer.

18. The imaging system of any of claims 1 to 17, further comprising:
an illumination light source;
illumination light timing circuitry to generate discrete light pulses using the illumination light source; and
image sensor timing circuitry to time-gate the array of sensor pixels based on the discrete light pulses of the illumination light.

~~19. The imaging system of claim 1, wherein the analysis substrate further comprises a spacer layer between the localization layer and the sensor layer, the spacer layer having a thickness based on the propagation of fluorescence from the fluorescent dye.~~

~~20. The imaging system of claim 19, wherein the spacer layer comprises a planar waveguide.~~

~~21. The imaging system of claim 19 or 20, wherein the thickness of the spacer layer corresponds to the propagation of fluorescence from the fluorescent dye being received by a predefined proportion of the array of sensor pixels.~~

~~22. The imaging system of claim 19 or 20, wherein the thickness of the spacer layer is selected based on a predefined full width at half maximum of the propagation of fluorescence from the fluorescent dye.~~

~~23. The imaging system of claim 1, wherein the imaging circuitry further comprises restriction circuitry restricting the fitting circuitry to fit the function to the pixel intensity distribution only in at least one area indicated by a well distribution pattern.~~

~~24. The imaging system of claim 23, wherein the imaging circuitry further comprises rejection circuitry to reject at least one localization outside the area indicated by the well distribution pattern.~~

~~25. The imaging system of any of claim 1, further comprising:
an illumination light source;
illumination light timing circuitry to generate discrete light pulses using the illumination light source; and
image sensor timing circuitry to time-gate the array of sensor pixels based on the discrete light pulses of the illumination light.~~

~~26.19.~~ An analysis substrate comprising:
a localization layer to be provided with a sample comprising a nucleotide provided with a fluorescent dye; and
a sensor layer comprising an array of sensor pixels, the localization layer being on-chip relative to the sensor layer, one or more of the array of sensor pixels to receive a propagation of fluorescence from the fluorescent dye.

~~27.20.~~ The analysis substrate of claim ~~26~~19, wherein the analysis substrate is part of a flowcell.

~~28.21.~~ The analysis substrate of any of claims ~~26~~19 or ~~27~~20, wherein the localization layer comprises nanowells.

~~29.22.~~ The analysis substrate of any of claims ~~26~~19 or ~~27~~20, wherein the localization layer comprises a zero-mode waveguide.

~~30.23.~~ The analysis substrate of any of claims ~~26~~19 to ~~29~~22, wherein the localization layer comprises a surface passivation layer.

~~31.24.~~ The analysis substrate of any of claims ~~26~~19 to ~~30~~23, wherein the sensor layer comprises a complementary metal-oxide-semiconductor chip.

~~32.25.~~ The analysis substrate of any of claims ~~26~~19 to ~~30~~23, wherein the array of sensor pixels comprises an array of photodiodes.

~~33.26.~~ The analysis substrate of any of claims ~~26~~19 to ~~30~~23, wherein the sensor layer

comprises a charge-coupled device.

~~34.27.~~ The analysis substrate of any of claims ~~2619~~ to ~~3326~~, further comprising a spacer layer between the localization layer and the sensor layer, the spacer layer having a thickness based on the propagation of fluorescence from the fluorescent dye.

~~35.28.~~ The analysis substrate of claim ~~3427~~, wherein the spacer layer comprises a planar waveguide.

~~36.29.~~ The analysis substrate of any of claims ~~3427~~ to ~~3528~~, wherein the thickness of the spacer layer corresponds to the propagation of fluorescence from the fluorescent dye being received by a predefined proportion of the array of sensor pixels.

~~37.30.~~ The analysis substrate of any of claims ~~3427~~ to ~~3629~~, wherein the thickness of the spacer layer is selected based on a predefined full width at half maximum of the propagation of fluorescence from the fluorescent dye.

~~38.31.~~ The analysis substrate of any of claims ~~2619~~ to ~~3730~~, wherein the sensor layer provides a single field of view of the localization layer.

~~39.32.~~ The analysis substrate of any of claims ~~2619~~ to ~~3831~~, further comprising a filter layer between the localization layer and the sensor layer, the filter layer including at least one color filter.

~~40.33.~~ The analysis substrate of any of claims ~~2619~~ to ~~3932~~, wherein the analysis substrate does not have a filter between the localization layer and the sensor layer, and wherein the analysis substrate does not have a waveguide between the localization layer and the sensor layer.

~~41.—A method comprising:~~
~~performing on-chip imaging of a sample at an analysis substrate; and~~
~~applying single-molecule localization microscopy (SMLM) to at least one image of the on-chip imaging.~~

~~42.—The method of claim 41, wherein:~~

~~performing the on-chip imaging comprises:~~

~~applying the sample to a localization layer of the analysis substrate, the sample comprising a nucleotide provided with a fluorescent dye;~~

~~receiving a propagation of fluorescence from the fluorescent dye using at least one sensor pixel at a sensor layer of the analysis substrate, the sensor layer comprising an array of sensor pixels; and~~

~~performing the SMLM comprises:~~

analyzing the sample based on the propagation of fluorescence from the fluorescent dye.

43.—The method of claim 42, wherein analyzing the sample comprises identifying, using a signal of the sensor layer, a pixel intensity distribution due to a point spread function, and determining a centroid of the pixel intensity distribution by fitting a function to the pixel intensity distribution.

44.—The method of claim 43, further comprising restricting the fitting to at least one area indicated by a well distribution pattern for the localization layer.

45.—The method of claim 44, further comprising rejecting at least one localization outside the area indicated by the well distribution pattern.

46.—The method of any of claims 42 to 45, further comprising introducing a photoswitching buffer at the localization layer before receiving the propagation of fluorescence, wherein the propagation of fluorescence is generated by a photoswitching dye.

47.—The method of any of claims 42 to 45, wherein an element of the sample comprises a sticky element, and wherein the propagation of fluorescence is generated by a fluorescent dye that becomes at least temporarily attached to the sample by way of the sticky element.

48.—The method of any of claims 42 to 45, wherein receiving the propagation of fluorescence from the fluorescent dye using the at least one sensor pixel comprises imaging a fluorescent dye based on the fluorescent dye becoming incorporated into the sample.

49.—The method of any of claims 41 to 45, wherein a fluorescent dye is attached to a nucleotide by a phosphate tail, and wherein the phosphate tail is cleaved during incorporation of the nucleotide into the sample.

50.—The method of claim 49, wherein a blocker on the nucleotide prevents further incorporation.

51.—The method of any of claims 46 to 50, wherein performing the SMLM comprises performing sequencing by synthesis.

52.—The method of any of claims 41 to 51, wherein the sample comprises single molecules.

53.—The method of any of claims 41 to 51, wherein the sample comprises a cluster of molecules.

54.—The method of any of claims 42 to 53, wherein the on-chip imaging comprises a single field of view of the localization layer.

55.—The method of any of claims 41 to 54, wherein the on-chip imaging and the

SMLM are performed as part of sequencing of the sample.

56.—The method of any of claims 42 to 55, further comprising performing color filtering of the propagation of fluorescence.

57.—The method of any of claims 42 to 56, further comprising shaping the propagation of fluorescence to have a predefined full width at half maximum at the sensor layer.

58.—The method of claim 57, wherein shaping the propagation of fluorescence includes providing a spacer layer between the localization layer and the sensor layer.

59.—The method of any of claims 42 to 58, further comprising triggering the propagation of fluorescence by chemiluminescence.

60.—The method of claim 59, wherein triggering the propagation of fluorescence comprises catalyzing a light emitting compound.

61.—The method of claim 60, wherein the light emitting compound comprises D-luciferin or a luciferin derivative, wherein a catalyst comprises luciferase, the method further comprising introducing one or more cofactors.

62.—The method of any of claims 42 to 58, further comprising operating an illumination light source to trigger the propagation of fluorescence.

63.—The method of claim 62, wherein operating the illumination light source comprises generating discrete light pulses using the illumination light source.

64.—The method of claim 63, further comprising time-gating the sensor layer based on the discrete light pulses of the illumination light source.

65.—A method comprising:
setting at least one of an activation rate or a deactivation rate of fluorescent tags for a sample to be sequenced;
stochastically switching between active fluorophore sets and inactive fluorophore sets of the fluorescent tags;
imaging the sample using on-chip imaging;
performing localization of at least one centroid of a point spread function in at least one image; and

determining whether to adjust the activation rate or the deactivation rate.

66.—The method of claim 65, wherein the determination is based on whether a number of localizations has decreased.

67.—The method of any of claims 65 to 66, further comprising adjusting at least the activation rate based on the determination.

68.—The method of claim 67, wherein adjusting the activation rate comprises changing an illumination wavelength.

69.—The method of claim 67, wherein adjusting the activation rate comprises increasing a concentration of a chemical substance at the sample.

70.—The method of any of claims 65 to 69, further comprising triggering a propagation of fluorescence from the active fluorophore sets by chemiluminescence.

71.—The method of claim 70, further comprising catalyzing a light-emitting compound to trigger the propagation of fluorescence from the active fluorophore sets.

72.—The method of claim 71, wherein the light-emitting compound comprises D-luciferin or a luciferin derivative, wherein a catalyst comprises luciferase, the method further comprising introducing one or more cofactors.

73.—The method of any of claims 65 to 69, further comprising operating an illumination light source to trigger a propagation of fluorescence.

74.—The method of claim 73, wherein operating the illumination light source comprises generating discrete light pulses using the illumination light source.

75.—The method of claim 74, further comprising time-gating a sensor layer based on the discrete light pulses of the illumination light source.

76.—A method comprising:
conjugating a light-emitting compound with a nucleotide of a sample material at an analysis substrate;

introducing one or more cofactors at the analysis substrate;

introducing a catalyst at the analysis substrate;

performing on-chip imaging of the sample material at the analysis substrate; and

applying single-molecule localization microscopy (SMLM) to at least one image of the on-chip imaging.

77.—The method of claim 76, wherein the light-emitting compound comprises D-luciferin or a luciferin derivative, and the catalyst comprises luciferase.

78.—The method of any of claims 76 to 77, wherein the cofactor includes at least one of oxygen or adenosine triphosphate.

79.—The method of any of claims 76 to 78, wherein:

performing the on-chip imaging comprises:

applying the sample material to a localization layer of the analysis substrate,

the sample comprising a nucleotide provided with a fluorescent dye;

~~receiving a propagation of fluorescence from the light-emitting compound using at least one sensor pixel at a sensor layer of the analysis substrate, the sensor layer comprising an array of sensor pixels; and performing the SMLM comprises:~~

~~analyzing the sample material based on the propagation of fluorescence from the fluorescent dye.~~

~~80.—The method of claim 79, wherein analyzing the sample comprises identifying, using a signal of the sensor layer, a pixel intensity distribution due to a point spread function, and determining a centroid of the pixel intensity distribution by fitting a function to the pixel intensity distribution.~~