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Ultrasensitive 3D Nano plasmonic Microarray for Multiplex Detection of EGFR Mutations via Liquid Biopsy

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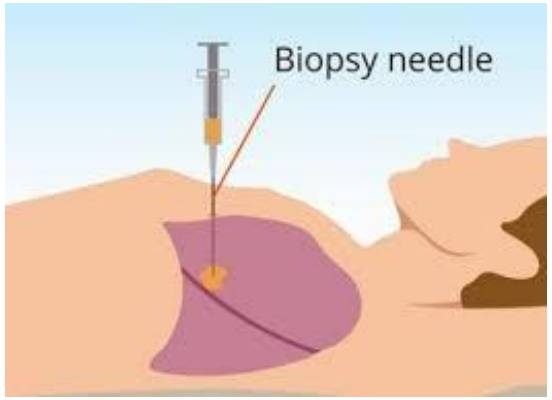
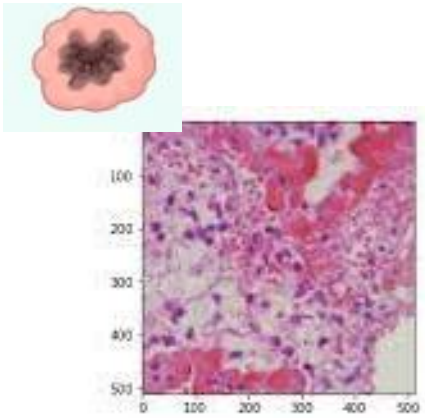
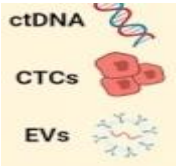
Cancer Liquid Biopsy







Tissue biopsy

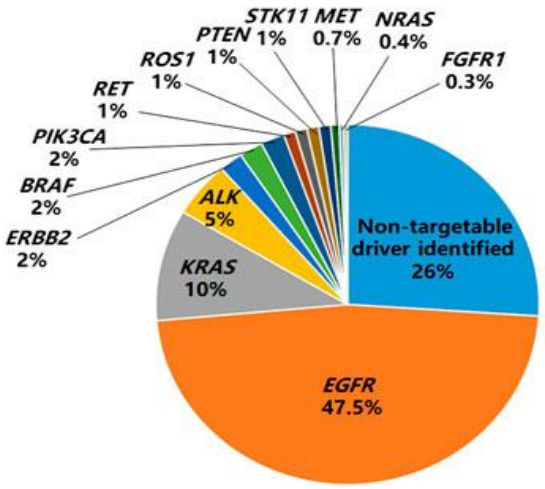
- Invasive
- Long duration required
- High sensitivity
- High cost of sample separation
- Lack of monitoring of tumor evolution
- No heterogeneity in tumor response

Liquid biopsy

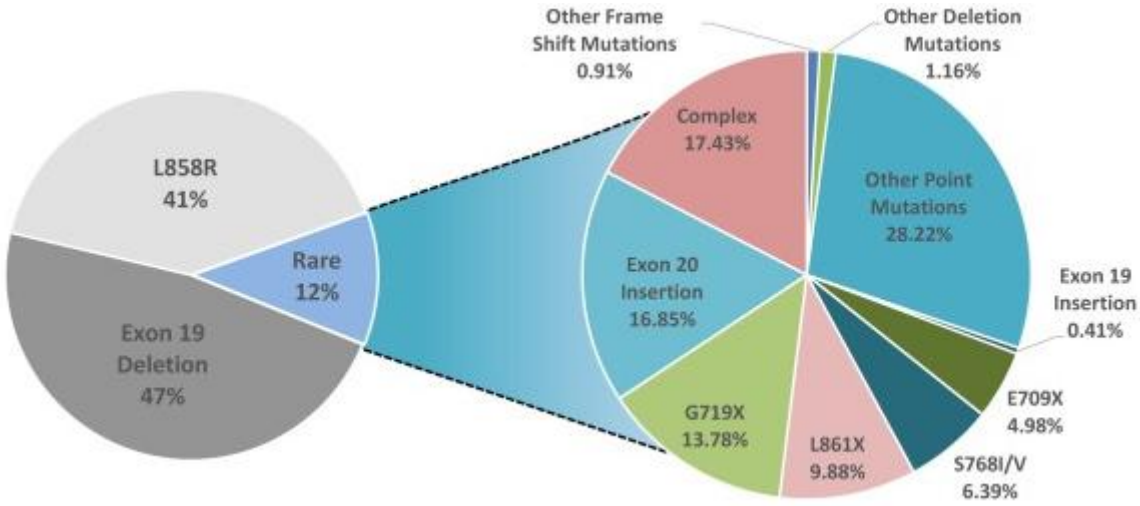
- Minimal invasiveness
- Short duration
- Low sensitivity
- Low sensitivity
- Real-time monitoring of drug response
- Heterogeneity in tumor response

The importance of EGFR mutations in non-small cell lung cancer patients

Mutation frequency in lung cancer patients



EGFR mutation frequency



Carcinoma	Biomarker	Targeted therapies
Non-small cell lung cancer	EGFR exon 19 deletions, EGFR exon 21 L858 alterations	erlotinib, afatinib, gefitinib
	EGFR exon 20 T790M alterations	Osimertinib
	ALK rearrangements	crizotinib, alectinib, certinib
	BRAF V600E	dabrafenib

EGFR: Epidermal growth factor receptor

ALK: Anaplastic lymphoma kinase

EGFR Exon 19 Deletion target detection

Representative Exon 19 deletion sequences

TGCTGGGCTCCGGTGCGTTCGGCACGGTGTATAAGGGACTCTGGATCCCAGAAGGTGAGAAAGTTAAAATTCCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCTCGATGAAGCCTACGTGATGGCCAGCGTGGACAACCCCCACGTGTGCCGCCTGCTGGGCATCTGCCTCACC

Wild type exon19 sequence, 200bp

Lung cancer

Exon 19 deletion

	2230	2240	2250	2260	2270
	TAAAATTCCCGTCGCTATCAAGGAATTAAGAGAAGCAACATCTCCGAAAGCCAACAAGGAAATCCT				
1	TAAAATTCCCGTCGCTATCAAGGAAT			CATCTCCGAAAGCCAACAAGGAAATCCT	
2	TAAAATTCCCGTCGCTATCAAGGAA			GCAACATCTCCGAAAGCCAACAAGGAAATCCT	
3	TAAAATTCCCGTCGCTATCAAGGA			TCCGAAAGCCAACAAGGAAATCCT	
4	TAAAATTCCCGTCGCTATCAA			AACATCTCCGAAAGCCAACAAGGAAATCCT	
5	TAAAATTCCCGTCGCTATCAAG			ACATCTCCGAAAGCCAACAAGGAAATCCT	
6	TAAAATTCCCGTCGCTATCAAGGAA			TCTCCGAAAGCCAACAAGGAAATCCT	
7	TAAAATTCCCGTCGCTATCAAGGAA			CCGAAAGCCAACAAGGAAATCCT	
8	TAAAATTCCCGTCGCTATCAAGG			CTCCGAAAGCCAACAAGGAAATCCT	
9	TAAAATTCCCGTCGCTATCAAGGAAT			CTCCGAAAGCCAACAAGGAAATCCT	
10	TAAAATTCCCGTCGCTATCAAGGAAT			CGAAAGCCAACAAGGAAATCCT	
11	TAAAATTCCCGTCGCTATCAAGGAA			CCAACATCTCCGAAAGCCAACAAGGAAATCCT	
12	TAAAATTCCCGTCGCTATCAAGGAA			CCATCTCCGAAAGCCAACAAGGAAATCCT	
13	TAAAATTCCCGTCGCTATCAAGG			TTCCGAAAGCCAACAAGGAAATCCT	
14	TAAAATTCCCGTCGCTATCAA			AATTCCGAAAGCCAACAAGGAAATCCT	
15	TAAAATTCCCGTCGCTATCAAGG			TATCTCCGAAAGCCAACAAGGAAATCCT	
16	TAAAATTCCCGTCGCTATCAAGGAA			CAGAAAGCCAACAAGGAAATCCT	
17	TAAAATTCCCGTCGCTATCAAGGAA			CCACCGAAAGCCAACAAGGAAATCCT	
18	TAAAATTCCCGTCGCTATCAAGG			TTGCTTCTCCGAAAGCCAACAAGGAAATCCT	
19	TAAAATTCCCGTCGCTATCAAGGA			GCAATCTCCGAAAGCCAACAAGGAAATCCT	
20	TAAAATTCCCGTCGCTATCAAGGA			GCCAACATCTCCGAAAGCCAACAAGGAAATCCT	
21	TAAAATTCCCGTCGCTATCAAGG			CATCTCCGAAAGCCAACAAGGAAATCCT	
22	TAAAATTCCCGTCGCTATCAAG			TCTCCGAAAGCCAACAAGGAAATCCT	
23	TAAAATTCCCGTCGCTATCAAG			AATTCCGAAAGCCAACAAGGAAATCCT	
24	TAAAATTCCCGTCGCTATCAA			AATATCTCCGAAAGCCAACAAGGAAATCCT	
25	TAAAATTCCCGTCGCTATCAA			AATTCCATCTCCGAAAGCCAACAAGGAAATCCT	
26	TAAAATTCCCGTCGCTATCAA			AATTCCAACATCTCCGAAAGCCAACAAGGAAATCCT	
27	TAAAATTCCCGTCGCTATCAAGGAATTAAGAGAAGCA				CCT
28	TAAAATTCCCGTCGCTATCAAGG			TCTCGAAAGCCAACAAGGAAATCCT	
29	TAAAATTCCCGTCGCTATCAAGGA			ATCTCCGAAAGCCAACAAGGAAATCCT	
	TAAAATTCCCGTCGCTATC			CAACATCTCCGAAAGCCAACAAGGAAATCCT	

Mutant type : Exon19 deletion sequences

cobas® EGFR Mutation Test v2

Exon 19	Ex19Del	1	2240_2251del12	6210
		2	2239_2247del9	6218
		3	2238_2255del18	6220
		4	2235_2249del15	6223
		5	2236_2250del15	6225
		6	2239_2253del15	6254
		7	2239_2256del18	6255
		8	2237_2254del18	12367
		9	2240_2254del15	12369
		10	2240_2257del18	12370
		11	2239_2248TTAAGAGAAG>C	12382
		12	2239_2251>C	12383
		13	2237_2255>T	12384
		14	2235_2255>AAT	12385
		15	2237_2252>T	12386
		16	2239_2258>CA	12387
		17	2239_2256>CAA	12403
		18	2237_2253>TTGCT	12416
		19	2238_2252>GCA	12419
		20	2238_2248>GC	12422
		21	2237_2251del15	12678
		22	2236_2253del18	12728
		23	2235_2248>AATTC	13550
		24	2235_2252>AAT	13551
		25	2235_2251>AATTC	13552
		26	2253_2276del24	13556
		27	2237_2257>TCT	18427
		28	2238_2252del15	23571
		29	2233_2247del15	26038

Number of possibilities, $2^{26} = 67,108,864$

Spectral Signal Enhancement through Plasmonic Nanomaterials

◆ Spectral Signal Amplification Technology Using Plasmonic Nanomaterials:

This technology uses plasmon resonance in noble metal nanomaterials triggered by light–electron interactions.

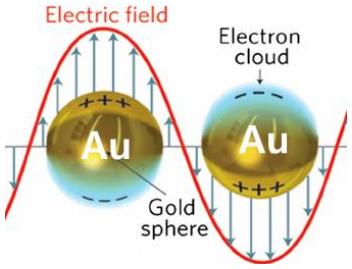
It greatly enhances Raman (SERS) and fluorescence (PEF) signals from surface-bound molecules.

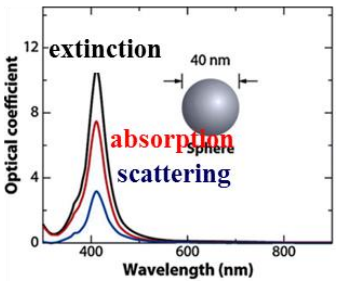
As a result, it allows ultra-sensitive detection of disease biomarkers at trace levels (below ppb) in biological samples.

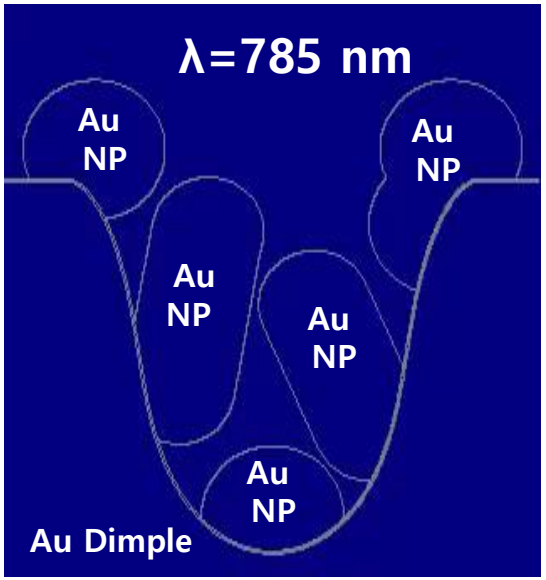
•**SERS:** Noble metal nanogaps where plasmonic coupling is maximized (SERS enhancement factor > 10^8)

•**PEF:** Nanogaps of ~10 nm, corresponding to the optimal distance for fluorescence signal generation (PEF enhancement factor ~ 10^2)

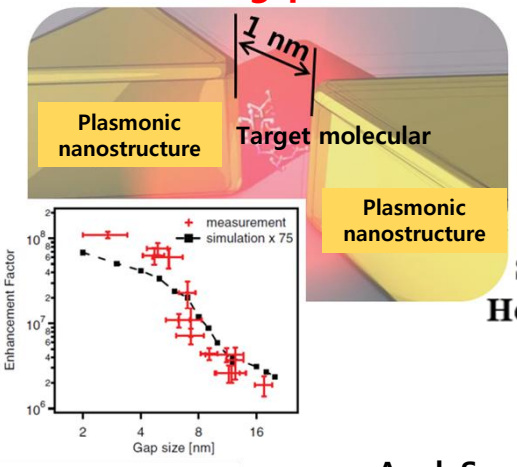
• LSPR and Plasmonic Hot-Volume

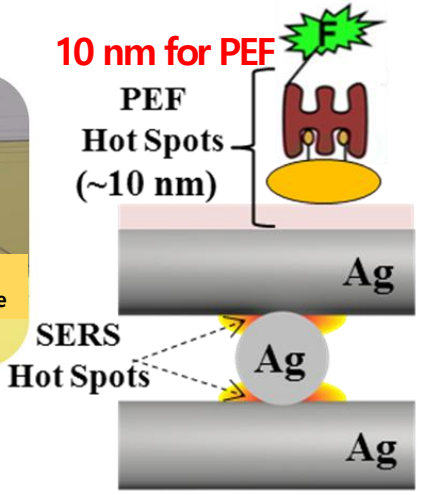






• SERS and PEF Hot Spots





Appl. Spectro. Rev., 2018, 1467440

*LSPR: Localized Surface Plasmon Resonance

*SERS: Surface-Enhanced Raman Spectroscopy

*PEF: Plasmon-Enhanced Fluorescence

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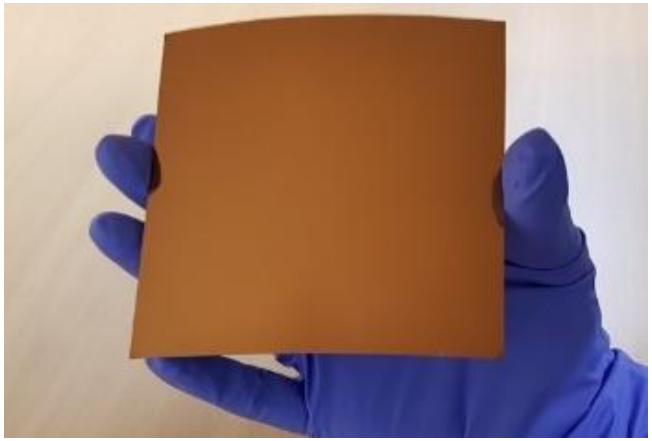
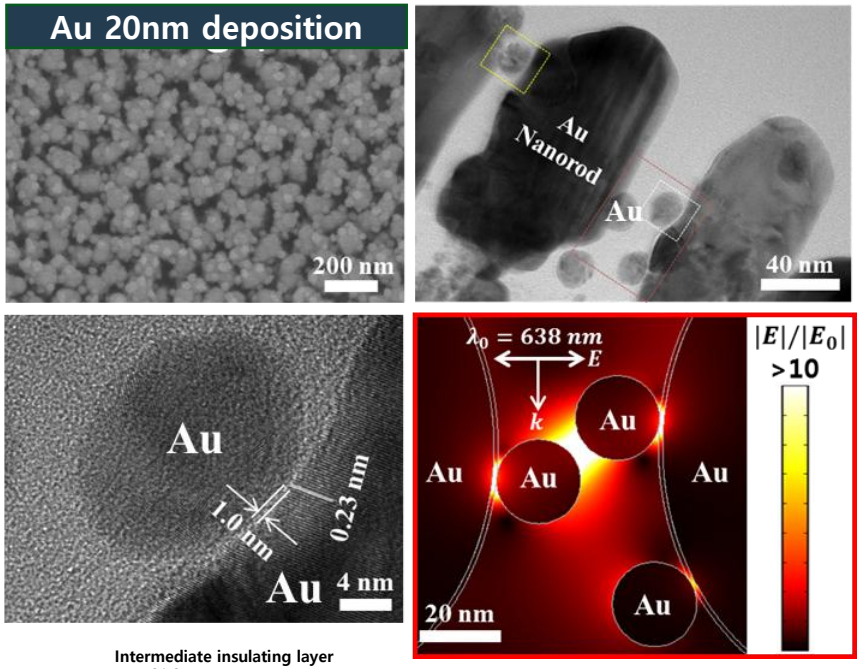
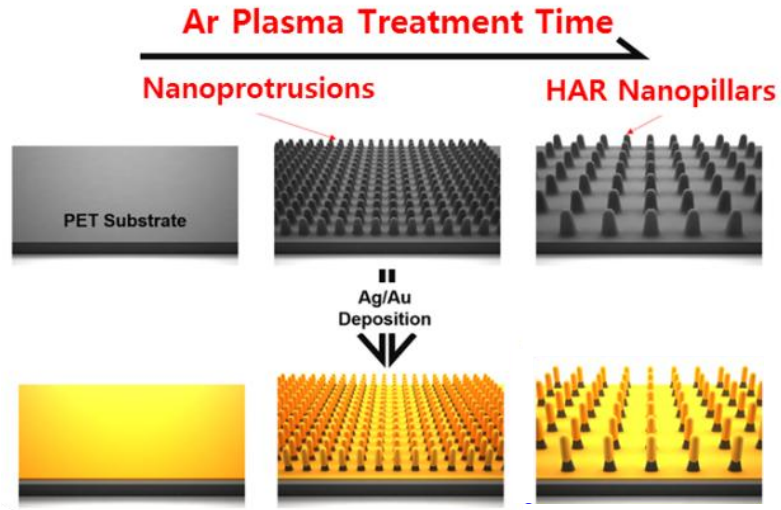
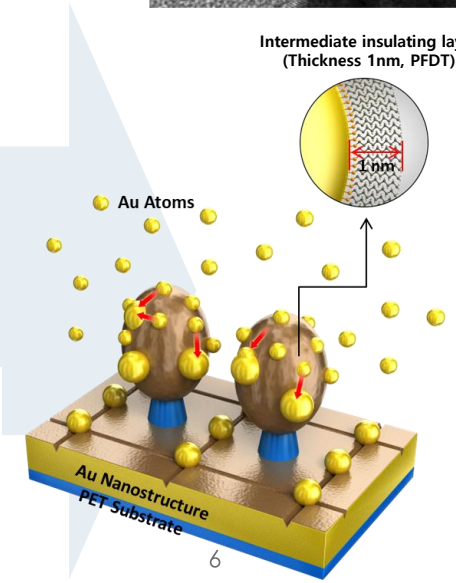
Large-Area, Ultra-Sensitive Nanoplasmonic Substrate

Surface-Enhanced Fluorescence Multilayer Nanomaterial





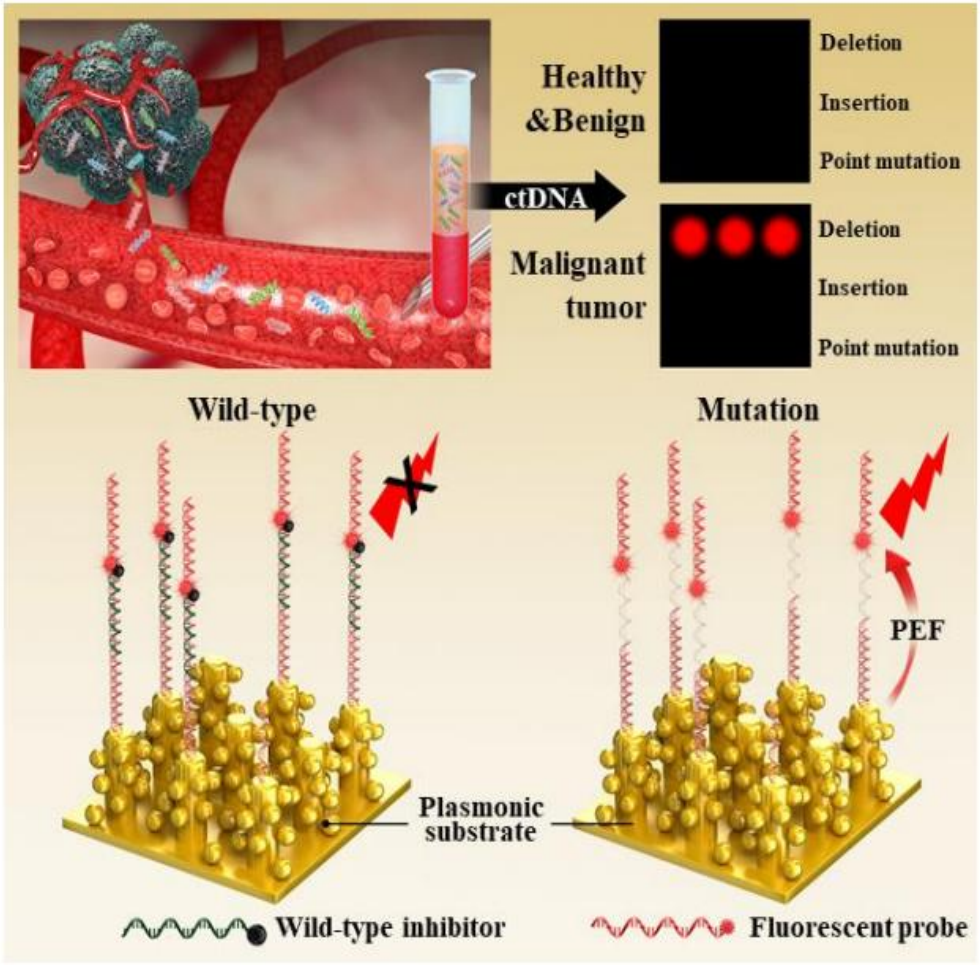


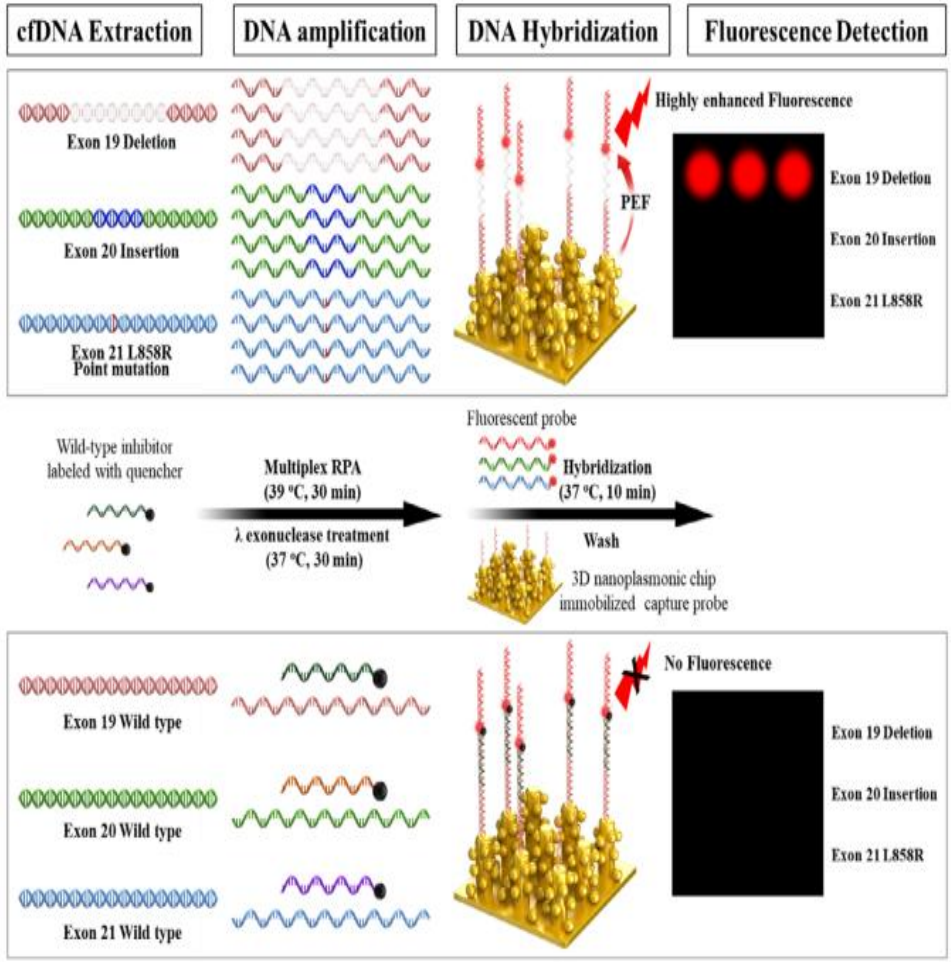


- Formation of Large-Area Nanopillars on Plastic Substrates
- Densely Packed Gold Nanoparticles on Gold Nanopillar Structures
- Scalable Large-Area Substrate Fabrication (> 4 inches)

Plasmonic microarray design and method

Small science (IF 12.7)
published inside cover





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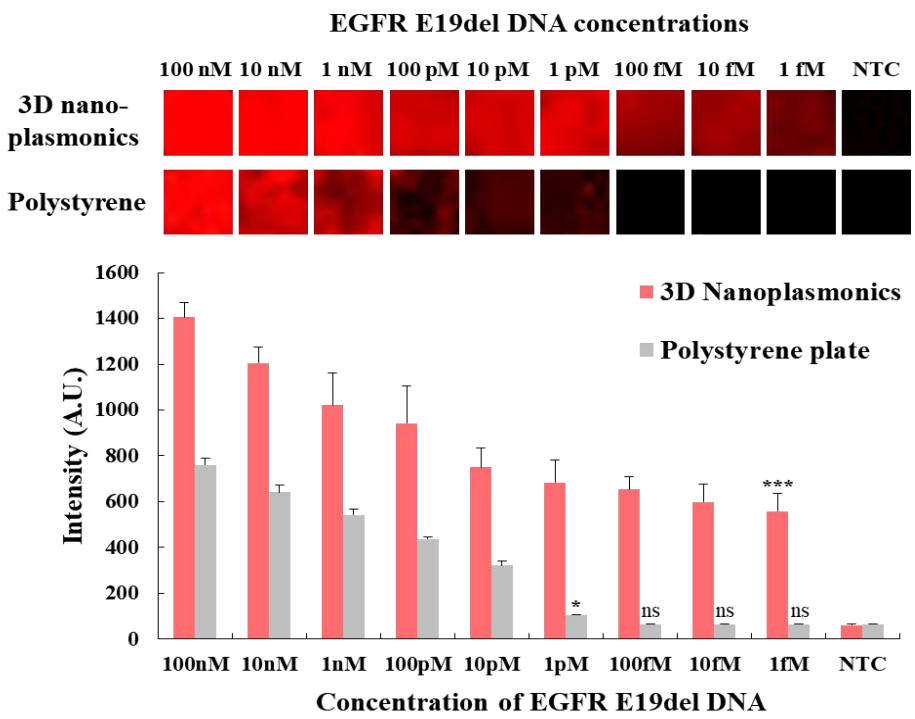
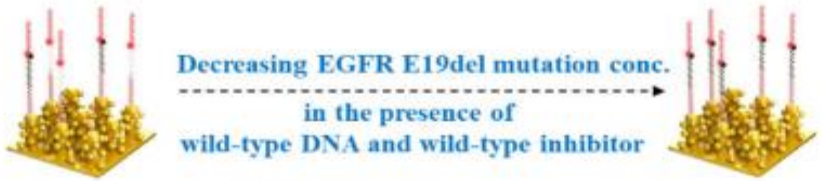
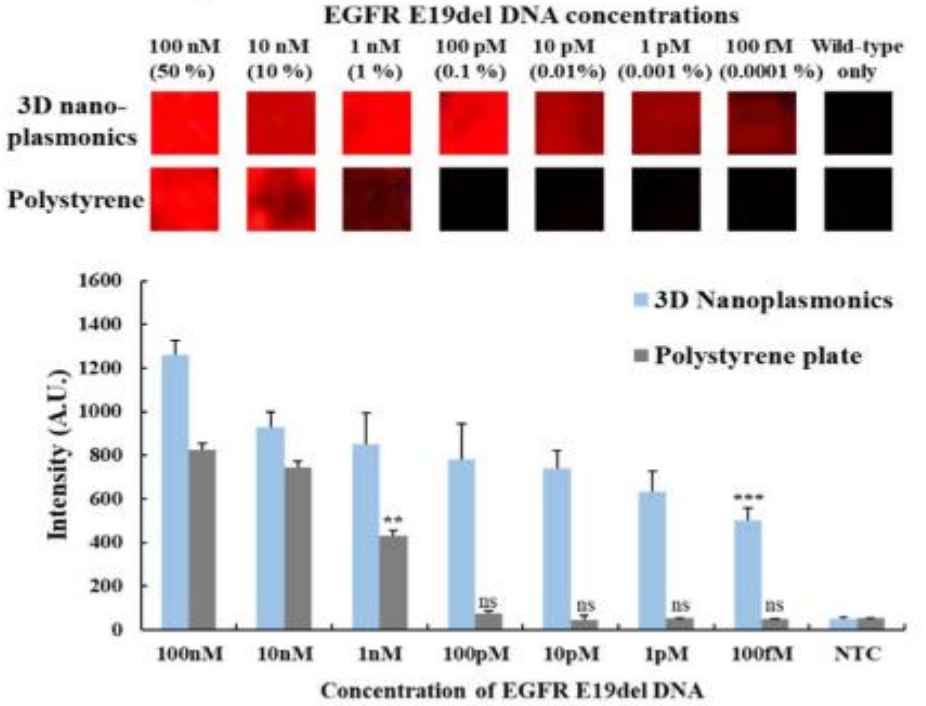
Detection sensitivity of plasmonic substrates (without nucleic acid amplification)

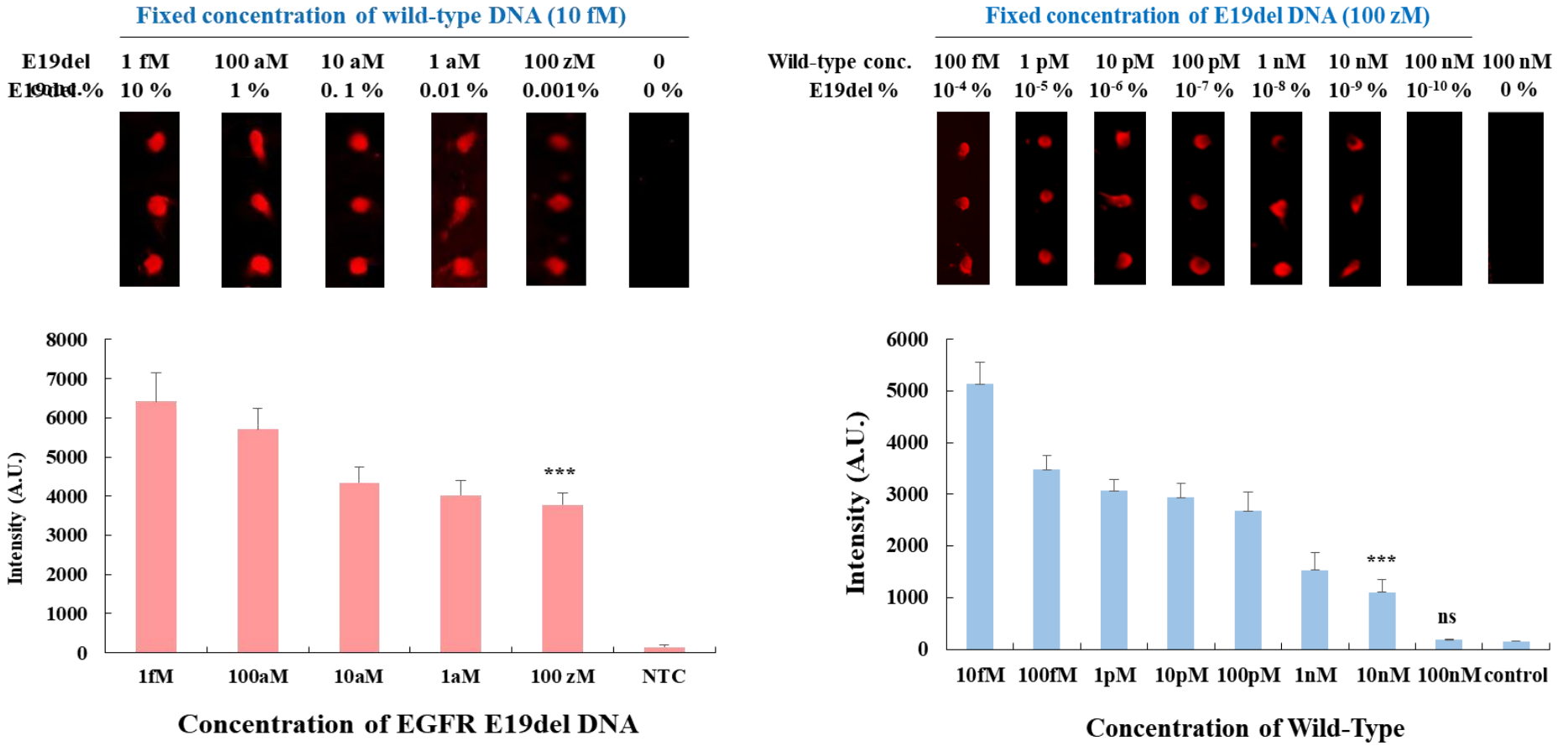






- Detection limit of mutant genes in a 3D plasmonic substrate without the normal gene: ~1 fM
- Detection of mutant genes at 0.0001% (100 fM) when the normal gene (100 nM) is present in a 3D plasmonic substrate

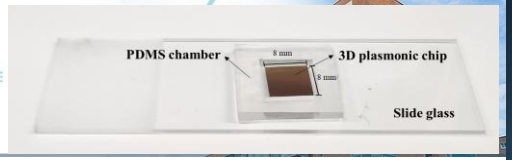
Plasmonic microarray analytical sensitivity (with nucleic acid amplification)

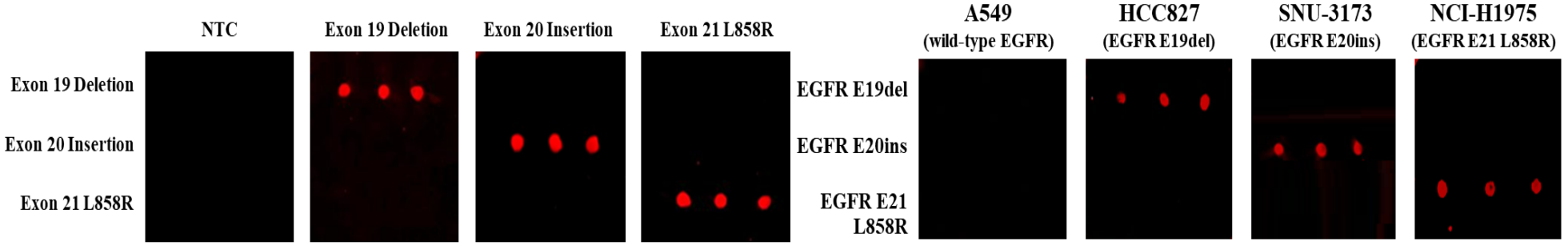


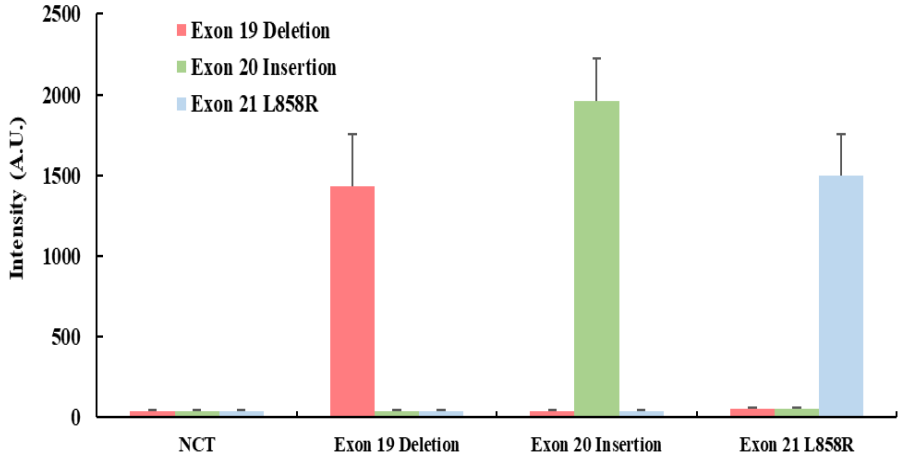
- **Detection limit of deletion mutant genes in a 3D plasmonic substrate without the normal gene**

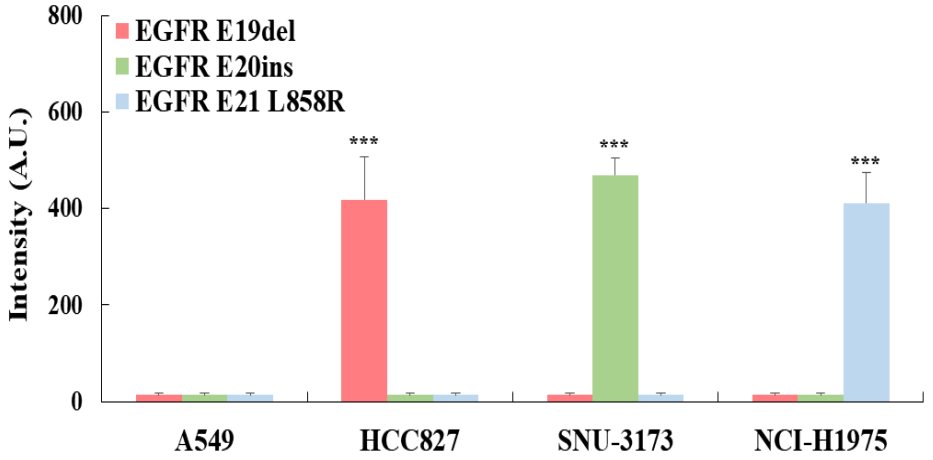
10^{-9}% Detection (100zM; 3 copies/rxn)

Plasmonic microarray analytical specificity







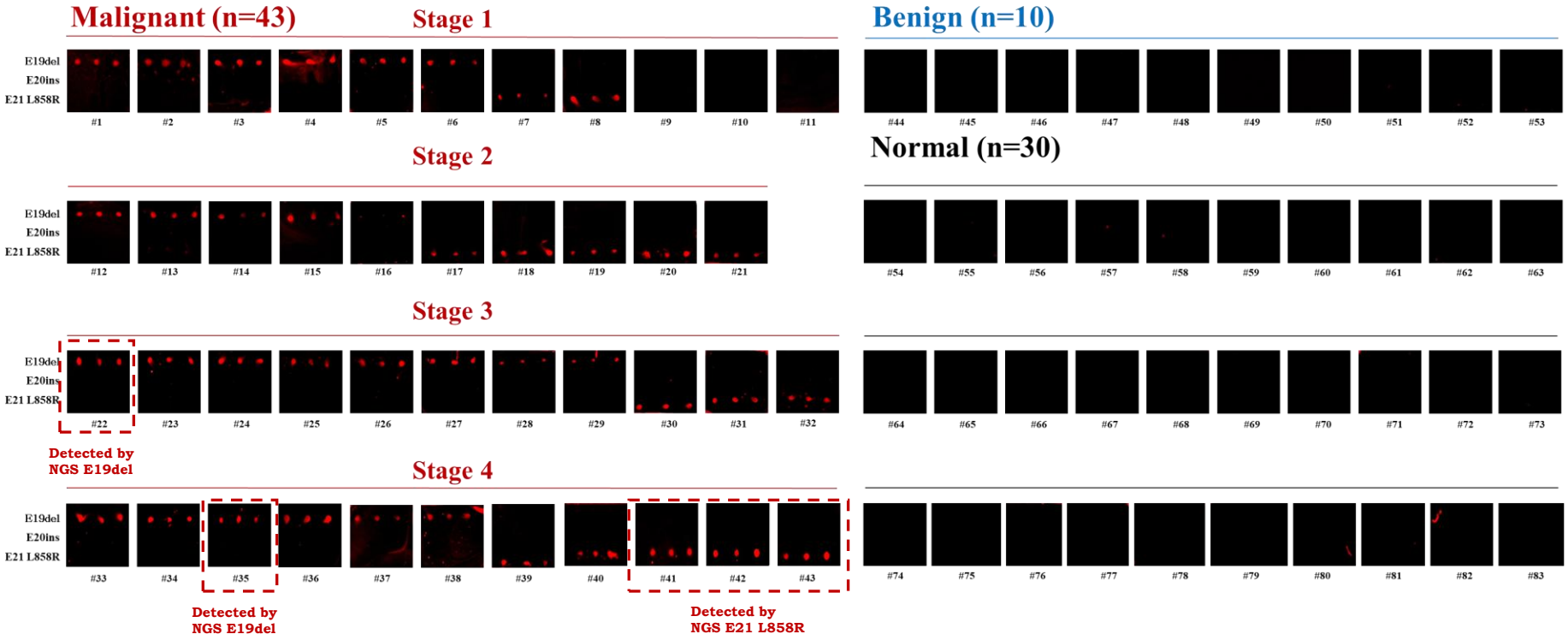


- 3D plasmonic microarray fabrication
  Exon 19 Deletion, Exon 20 Insertion, Exon 21 L858R point mutation 3-plex
  -> Fluorescent signal emission only at the specific mutation gene locations: ensuring high specificity.

Performance evaluation using clinical samples

Clinical sensitivity : 93%

Clinical Specificity: 100%



- Evaluation of 52 clinical samples (Malignant lung cancer n=21, Benign n=10, Normal n=21)
  -> Clinical sensitivity 93% Clinical specify 100% (agreement 100 % with NGS results)

Performance evaluation using clinical samples

Methods	Mutation frequency sensitivity (%)	Number of detectable deletions or insertions using one primer–probe set
Our assay	1×10^{-9}	All mutations in the target region (In this study, all deletions occurred within 26 bp and all insertions occurred within 45 bp)
Digital PCR	0.01	1
PNA-LNA PCR clamp	0.01	1
PCR invader	0.1	1
CastPCR	0.1	1
Real-time PCR	0.1	1
NGS	1	All mutations
Direct sequencing	10	All mutations

- Sensitivity improved by more than 10^7 times compared to existing EGFR mutation detection technologies
  Detection expected in clinical samples not detected by NGS

Conclusion

- In summary, we developed a 3D-nanoplasmonic-based EGFR mutation multiplex assay chip for detecting EGFR E19del, E20ins, and E21L858R point mutations with exceptional sensitivity.
- Compared to previously reported EGFR detection methods with a sensitivity range of 0.01%–1%, our approach achieved a superior higher sensitivity of 1 10^9 % mutant frequency due to the synergistic effects of the PEF of the 3D-nanoplasmonic and wild-type inhibitor.
- Based on this synergistic effect, clinical plasma ctDNA testing using the 3D-nanoplasmonic-based EGFR mutation multiplex assay chip not only diagnosed malignant tumors from stages 1 to 4, but also accurately distinguished benign and normal cases from malignant cases. As a result, it achieved 100% sensitivity and specificity.
- This method takes ≈70 min post-DNA extraction, and the total process takes around 2 h, including cfDNA extraction. This timeframe is shorter than that of the real-time PCR-based approach (cobas EGFR Mutation Test), which typically takes around 4 h.
- Our economical and effective rapid analysis method with high accuracy can aid in early cancer diagnostic screening, elimination of unnecessary tissue examinations, and monitoring of the therapeutic efficacy of EGFR-targeted treatments and cancer recurrence in clinical settings.
- The 3D-nanoplasmonic-based mutation multiplex assay chip, which functions as a microarray with the ability to immobilize various capture probes, can be readily applied for the detection of various cancer biomarkers.

Thank you for your attention