DNA Detection

This presentation is made possible through the following financial assistance award #70NANB23H276 awarded to Towson University from the U.S. Department of Commerce, National Institute of Standards and Technology (NIST)

Slides prepared by Kelly M. Elkins, Ph.D., Towson University, 2025

Background

In October 2023, Towson University was awarded a cooperative agreement from NIST to develop a standardized DNA training curriculum for the United States that addresses the components in ANSI/ASB Standard 115, *Standards for Training in Forensic Short Tandem Repeat Typing Methods Using Amplification, DNA Separation, and Allele Detection*. 2020. 1st Ed.

This presentation addresses the knowledge-based portion of the training program and covers the topic outlined in 4.2.3d in ANSI/ASB Standard 115.

Learning Objectives

1. history of DNA detection methods;
2. fluorescent dye detection;
    i. excitation,
    ii. emission;
3. dye-labeling of PCR primers;
4. computer software programs for DNA detection;
5. multicomponent analysis
i. spectral calibration
ii. spatial calibration;
6. analytical threshold;
7. fragment sizing and allele calling;
8. bins (including virtual bins);
9. limitations of the technology.

Terms & Definitions (ANSI/ASB 115)

- **Analytical threshold.** 1) The minimum height requirement at and above which detected peaks on a STR DNA profile electropherogram can be reliably distinguished from background noise; peaks above this threshold are generally not considered noise and are either artifacts or true alleles. 2) A “Relative Fluorescence Units” (RFU) level determined to be appropriate for use in the PCR/STR DNA typing process; a minimum threshold for data comparison is identified by the specific forensic laboratory through independent validation studies.
- **Artifact.** A non-allelic product of the amplification process (e.g., stutter, non-templated nucleotide addition, or other non-specific product), an anomaly of the detection process (e.g., pull-up or spike), or a byproduct of primer synthesis (e.g., “dye blob”) that may be observed on an electropherogram; some artifacts may complicate the interpretation of DNA profiles when they cannot be distinguished from the actual allele(s) from a particular sample.

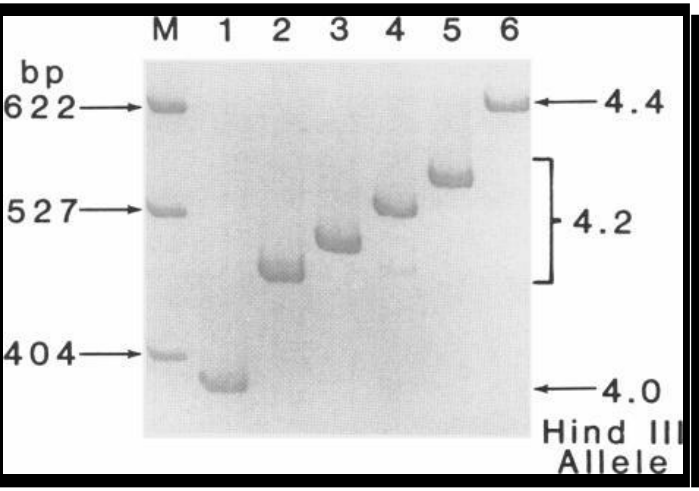
Terms & Definitions (ANSI/ASB 115)

- **Bin.** Allele designations corresponding to the window of fragment sizes for each allele, determined by empirical testing.
- **Spectral calibration.** An examination of the contribution of overlap in the emission spectrum of fluorescent dyes used for a specific DNA test on a capillary electrophoresis instrument; permits the color deconvolution necessary for multi-color STR typing or sequencing to be performed; a poor spectral calibration may cause artifact peaks or inaccurate peak height determinations.
- **Stochastic.** 1) Chance, or random variation 2) in DNA testing, refers to random sampling error from extracts containing low levels of DNA and/or random variation in selection of alleles amplified at a particular locus.

History of DNA Detection Methods

Mid 1980s to early 1990s

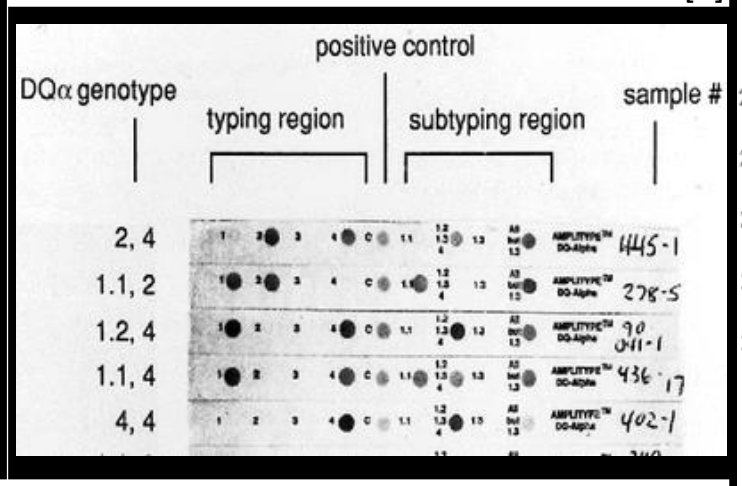
[1]



Restriction Fragment Length Polymorphism (RFLP) of Variable Number Tandem Repeat (VNTR) Minisatellites

Early to mid 1990s

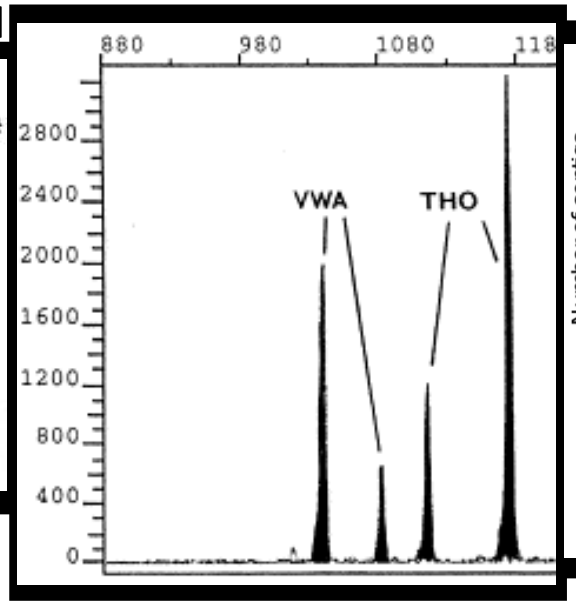
[2]



Reverse Dot Blot DNA Hybridization Polymarker (AmpliType HLA DQα1)

Mid 1990s-present

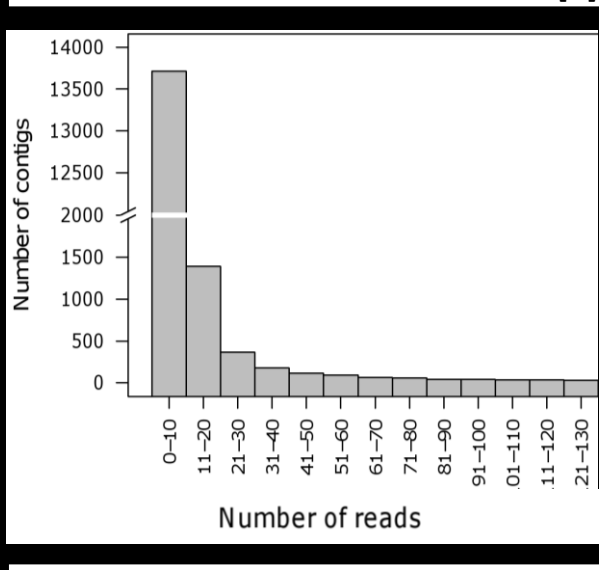
[3]



Short Tandem Repeat (STR) Microsatellites

1990s-present

[4]

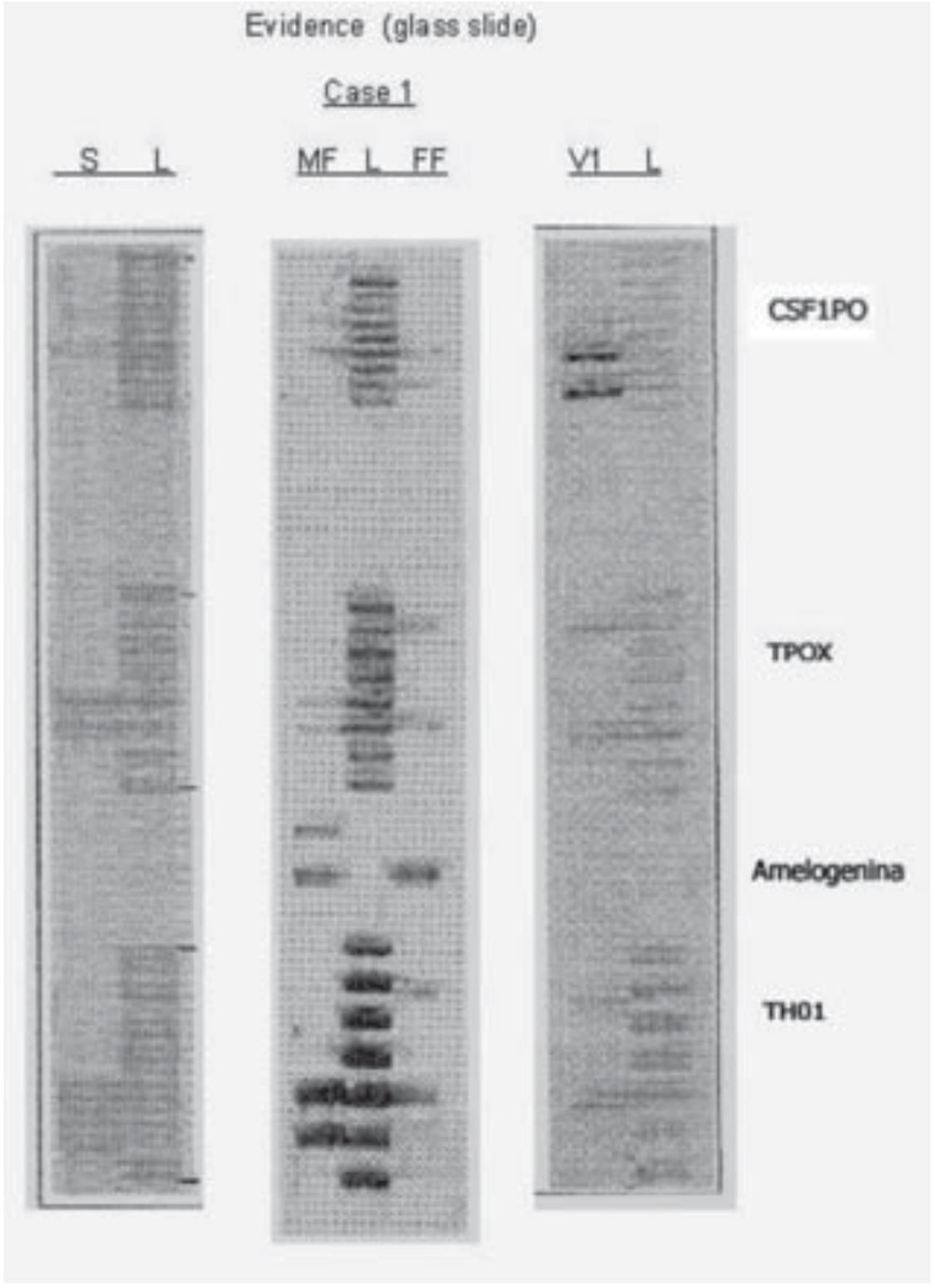


Single Nucleotide Polymorphism (SNP)

1. Goltsov, A. A., Eisensmith, R. C., Konecki, D. S., Lichter-Konecki, U., & Woo, S. L. (1992). Associations between mutations and a VNTR in the human phenylalanine hydroxylase gene. American journal of human genetics, 51(3), 627–636.
2. Walsh PS, Metzger DA, Higuchi R. Chelex 100 as a Medium for Simple Extraction of DNA for PCR-Based Typing from Forensic Material. BioTechniques. 2013;54(3):134–9.
3. Clayton TM, Whitaker JP, Sparkes R, Gill P. Analysis and interpretation of mixed forensic stains using DNA STR profiling. Forensic science international. 1998;91(1):55–70.
4. Helyar SJ, Limborg MT, Bekkevold D, Babbucci M, van Houdt J, Maes GE, et al. SNP Discovery Using Next Generation Transcriptomic Sequencing in Atlantic Herring (Clupea harengus). PloS one. 2012;7(8):e42089.

History of STR Typing: Detection

- First STR kit introduced targeted TH01 in1993 (Promega) with gel separation and silver stain detection
- CTT STR DNA typing kit (Promega, shown at right) was introduced in 1994
  - CTT refers to the multiplex of three STRs: CSF1PO, TPOX, TH01
- Silver stain
  - Silver binds to negatively charged DNA strands
  - Reduce silver ions (Ag^{+}) from silver nitrate to metallic silver (Ag^{0}) by alkaline formaldehyde
  - LOD 2.5 ng (5x EtBr stain)
  - Linear range 5-30 ng

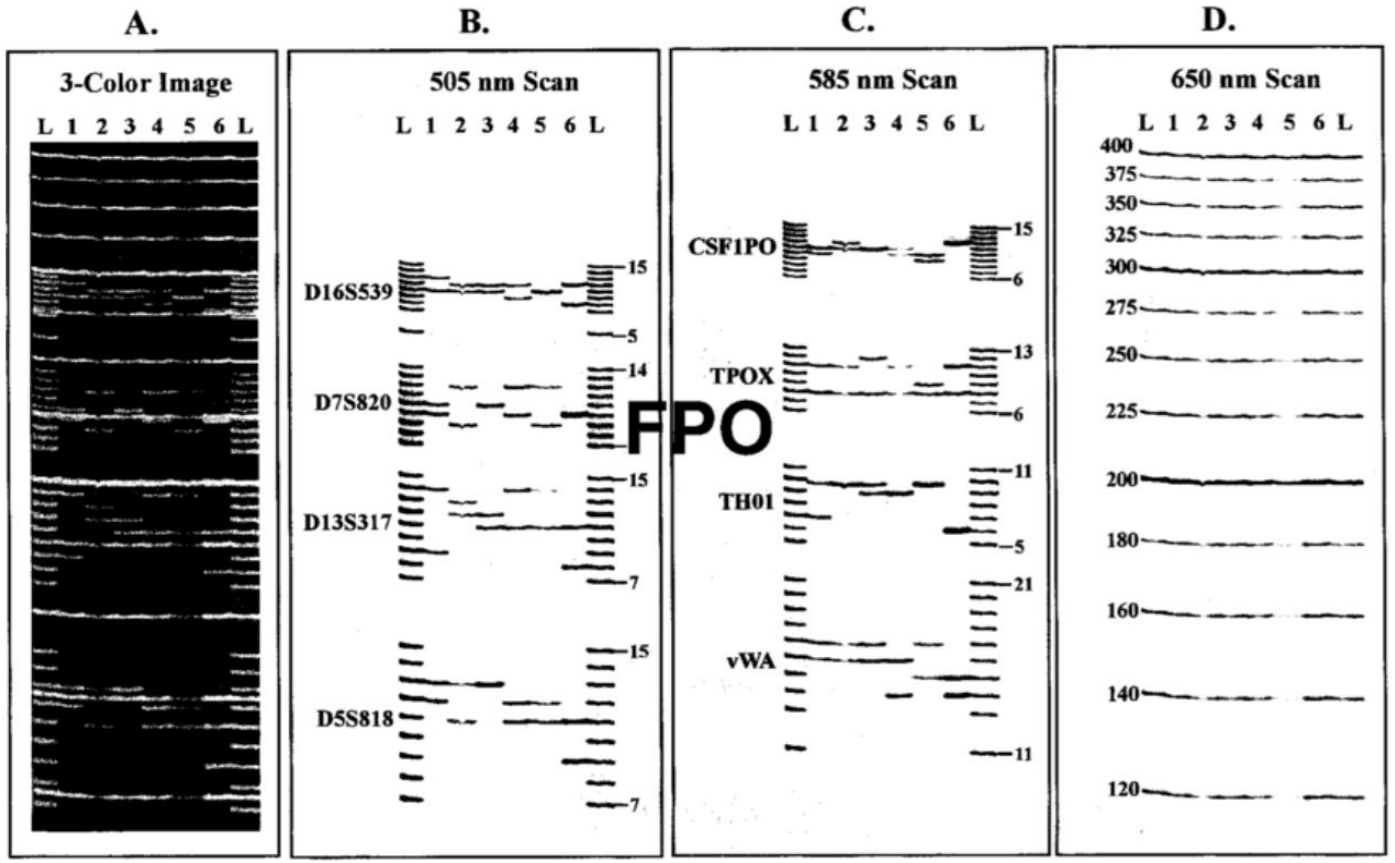


Gottlieb M, Chavko M. Silver staining of native and denatured eucaryotic DNA in agarose gels. Anal Biochem. 1987 Aug 15;165(1):33-7. doi: 10.1016/0003-2697(87)90197-7.

da Silva DA, Góes AC, de Carvalho JJ, de Carvalho EF. DNA typing from vaginal smear slides in suspected rape cases. Sao Paulo Med J. 2004 Mar 4;122(2):70-2. doi: 10.1590/s1516-31802004000200008. Figure CC BY 4.0 license

History of DNA Detection

- Detection using fluorescent dyes was introduced in 1996 in which different dyes were covalently bound to PCR primers for different loci
- Large polyacrylamide gels
  - PowerPlex™ System (Promega)
    - 8 loci using 3 dyes
      - Fluorescein (green)
      - TMR (red)
      - CXR (blue)
  - AmpFlSTR® Blue (Applied Biosystems)
    - 3 loci using 1 dye

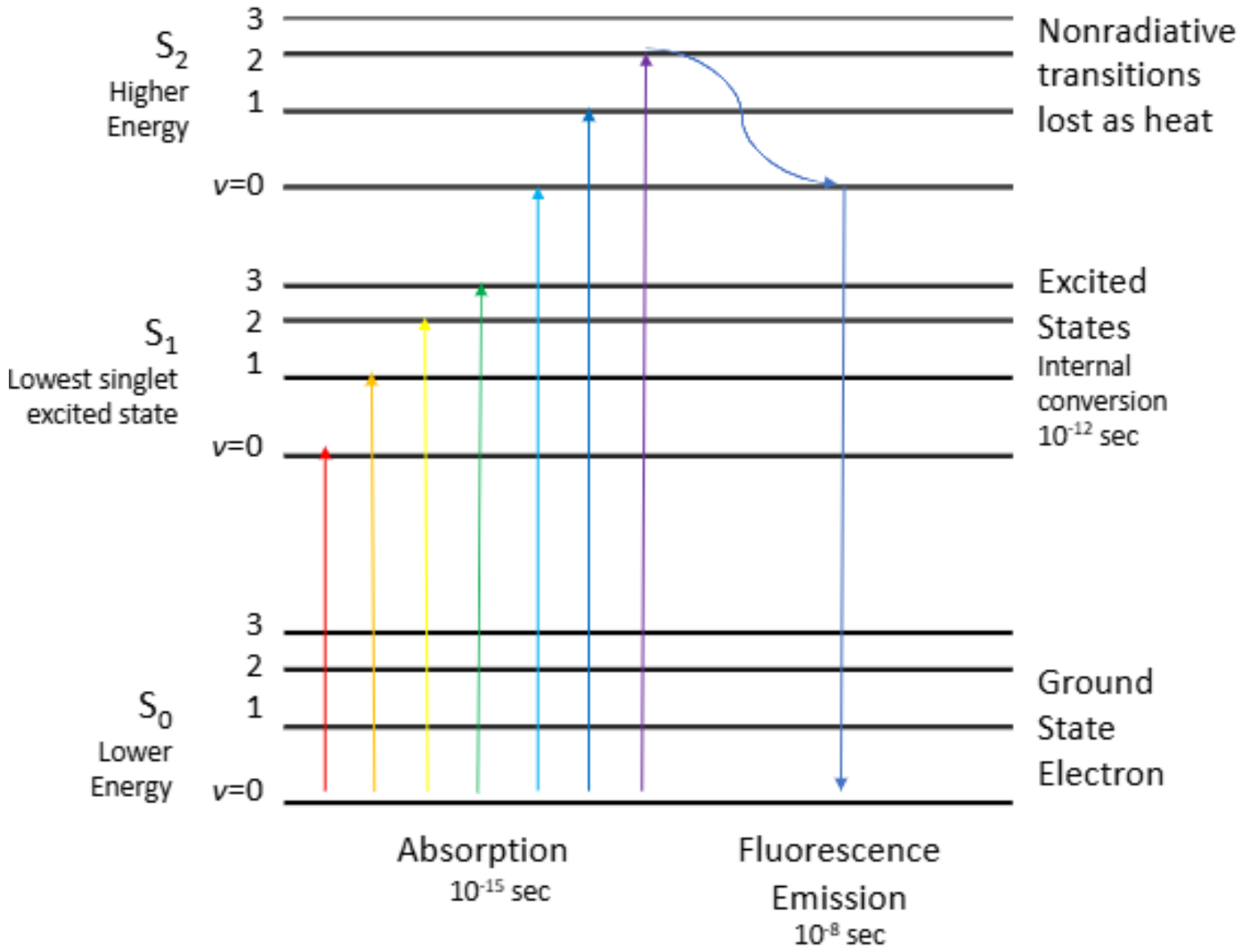


Lins, A., Micka, K., Sprecher, C., Taylor, J., Bacher, J., Rabbach, D., Bever, R., Creacy, S., and Schumm, J. "Development and Population Study of an Eight-Locus Short Tandem Repeat (STR) Multiplex System." ASTM International. *J. Forensic Sci.*. November 1998; 43(6): 1168–1180. https://doi.org/10.1520/JFS14381J

Fluorescence Dye Detection

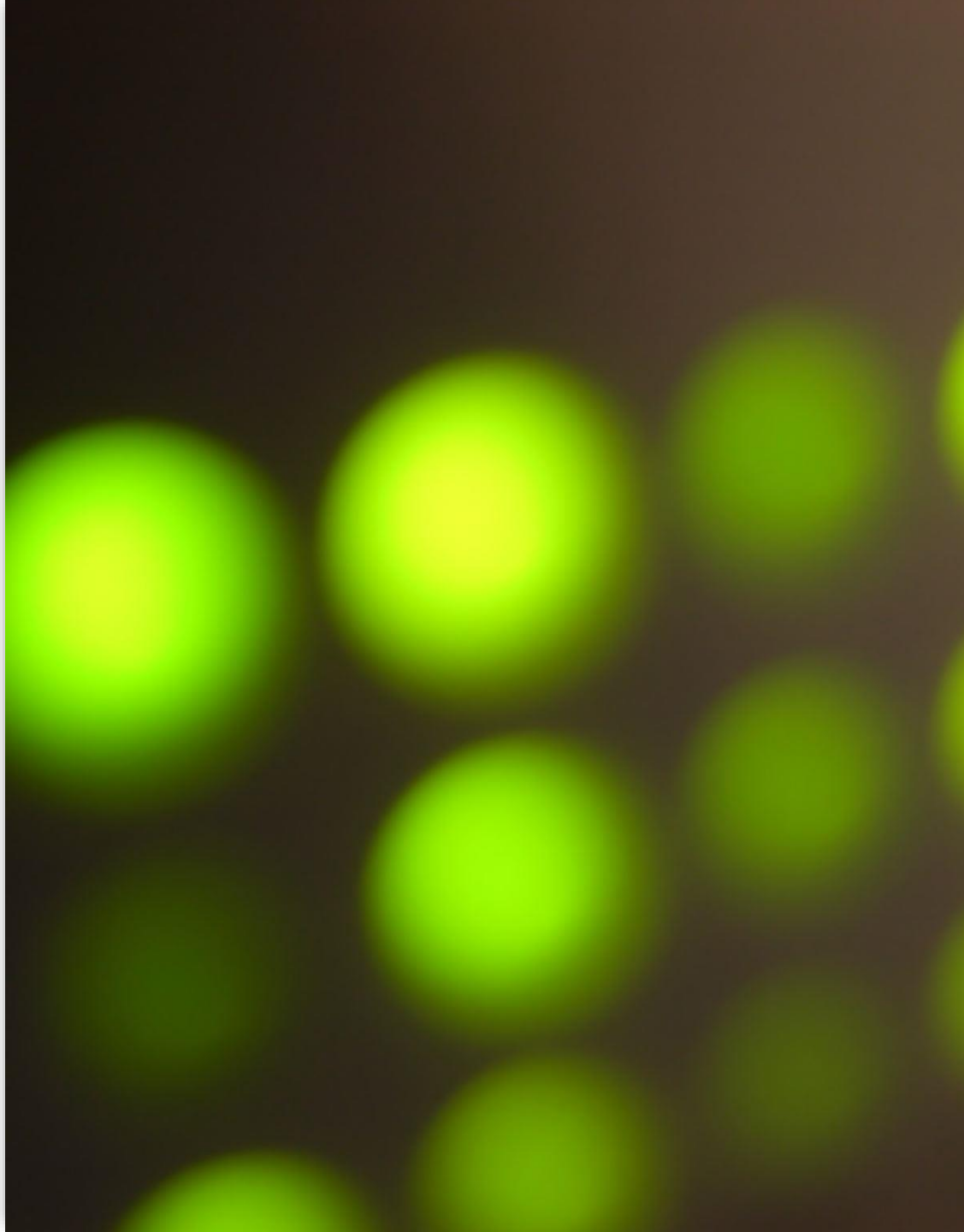
- **Excitation**- electron in fluorophore boosted by laser or LED energy to an excited state
- **Emission**- energy emitted by fluorophore as electron relaxes to ground state following nonradiative transitions resulting in a longer wavelength than excitation

Jablonski Diagram



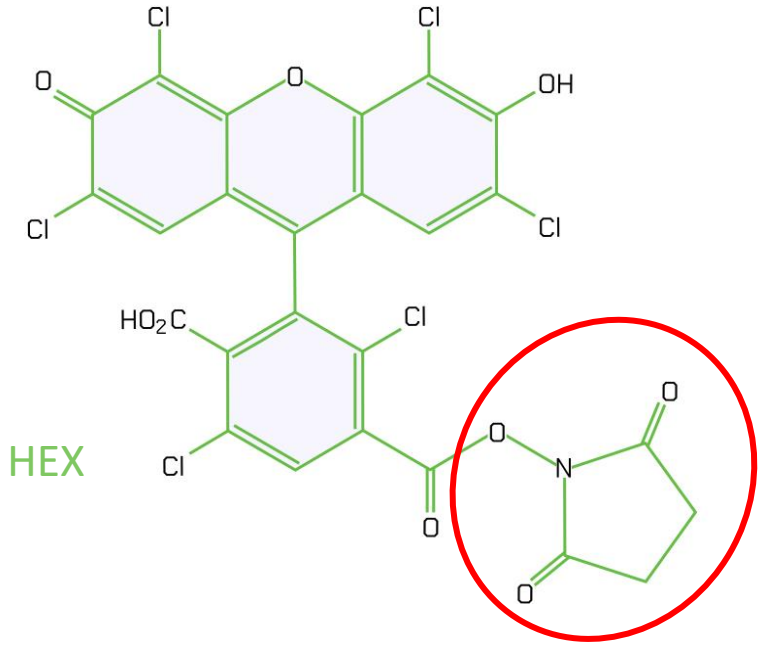
Fluorescence Detection

- Many molecules absorb UV-Vis radiation
- Some molecules fluoresce
- Fluorescent molecules (e.g., dyes) are used for DNA detection
  - Absorptions in 480-650 nm range
  - Emissions at longer wavelengths in the long UV or visible range
  - Peaks shift with pH, temperature, or concentration changes
- Light is emitted by fluorophore
  - Nanomolar detection limit
- Detected by fluorimeter or fluorescence spectrometer charge coupled device (CCD) detector

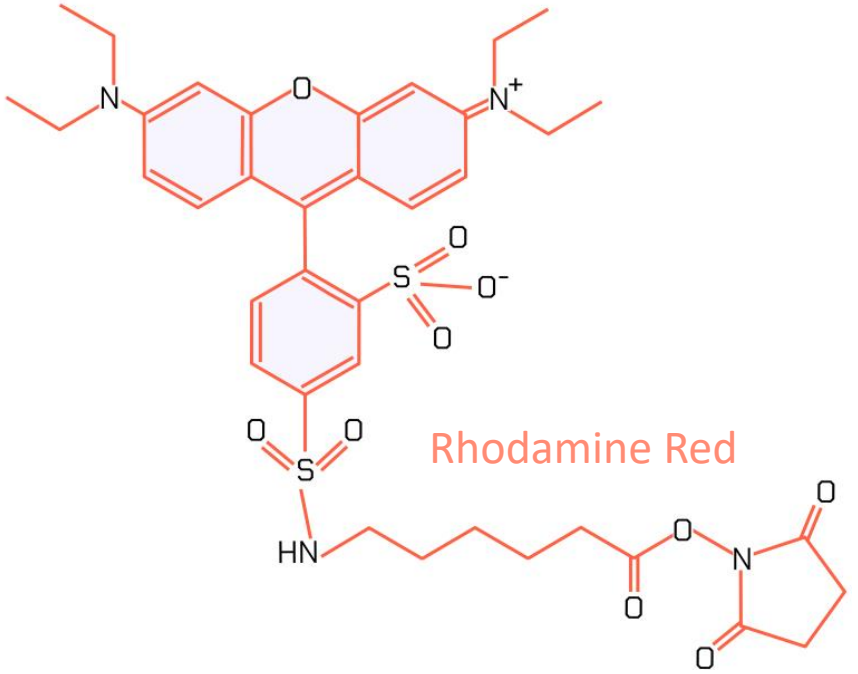


Fluorescent Dyes

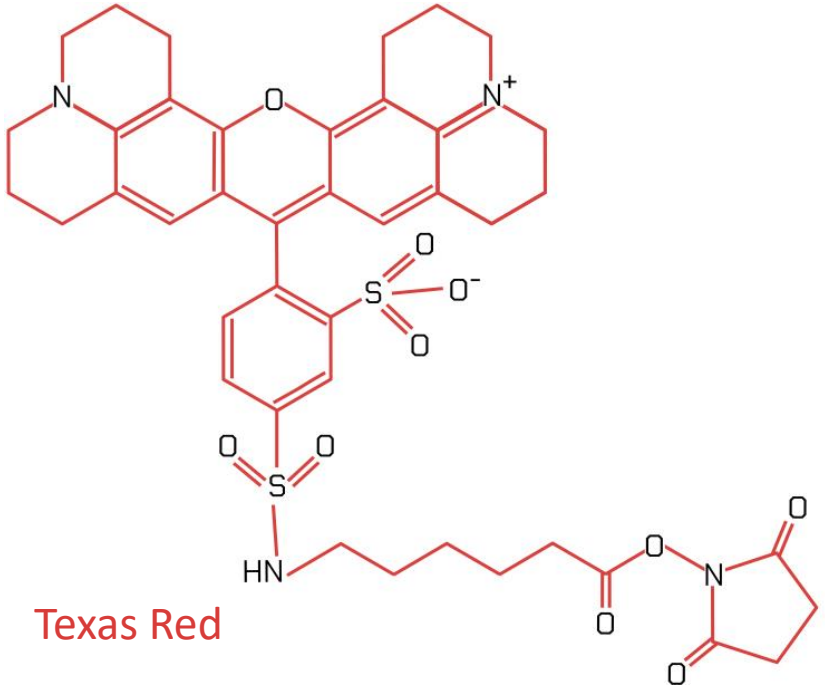
Dye are attached to the oligo at the succinimidyl ester (NHS ester)



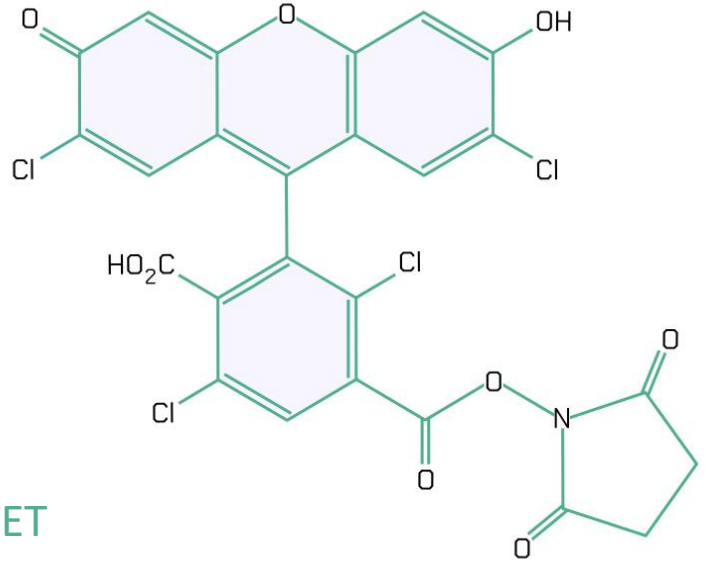
HEX



Rhodamine Red



Texas Red



TET

Fluorescent Dyes Emission Maxima

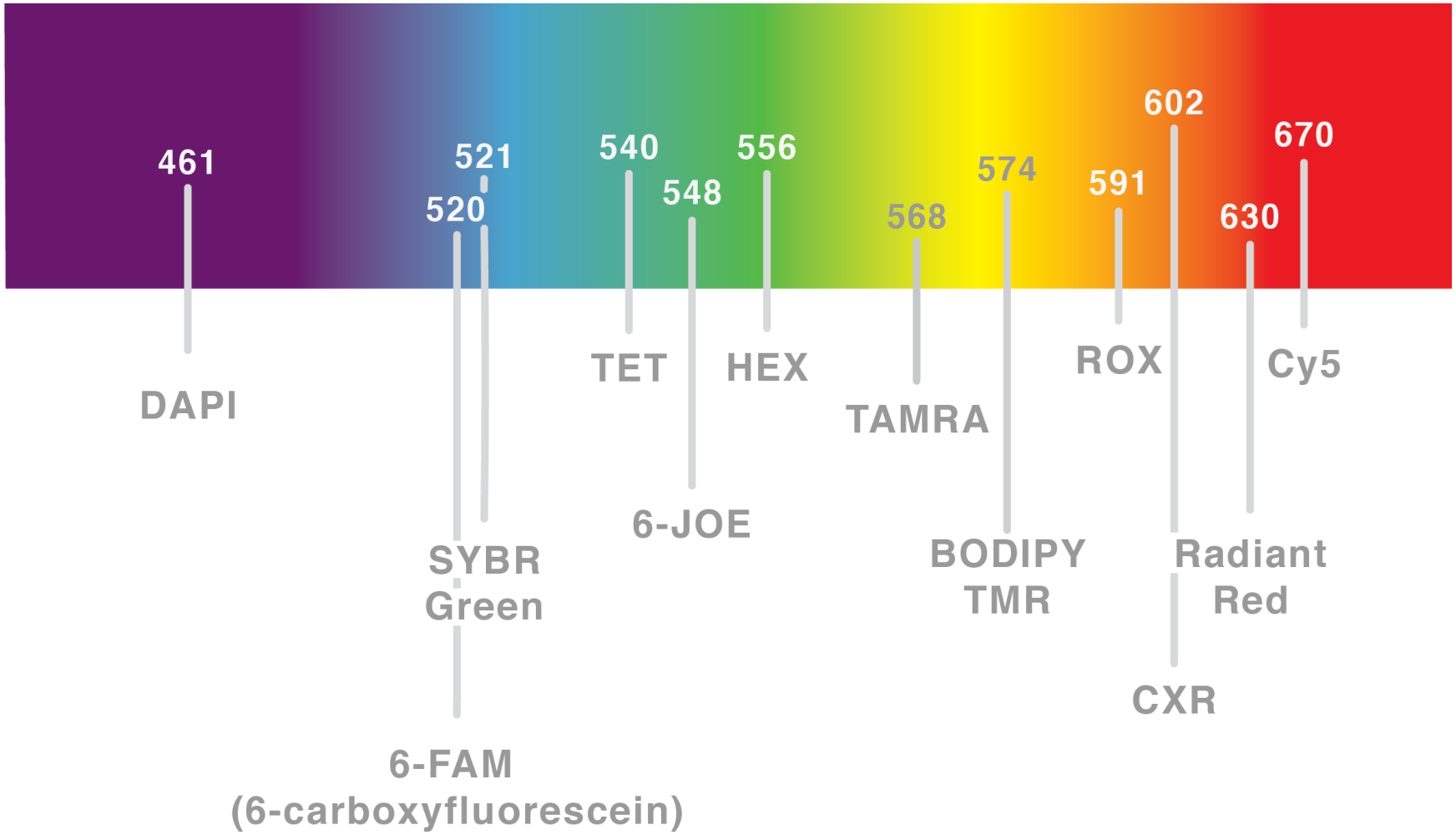


Figure credit: Anna-Sewell Killingstad

Absorption and emission maxima can be shifted to the red by additional π bonds and substituent groups

Fluorescent Dye Detection

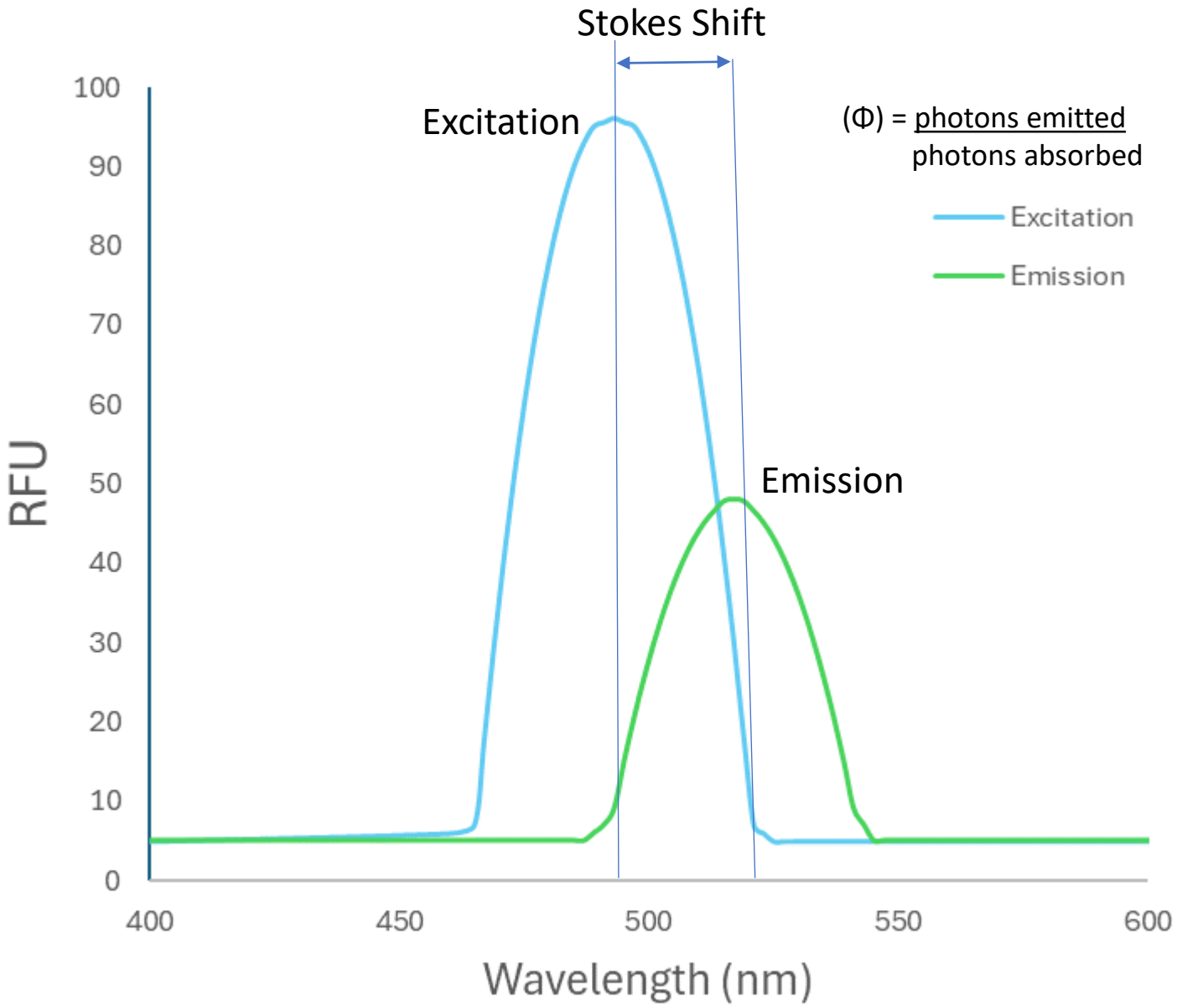
Dye	Emission Color	Excitation λ (nm)	Emission λ (nm)
DAPI	Purple	359	461
SYBR Green	Blue-Green	494	521
6-FAM (6-carboxyfluorescein)	Blue-Green	495	520
6-JOE	Green	520	548
TET	Green	520	540
HEX	Green-Yellow	530	556
BODIPY TMR	Orange	542	574
TAMRA	Yellow	542	568
ROX	Red	567	591
CXR	Red	580	602
Radiant Red	Red	495	630
Cy5	Red	650	670

Proprietary Dyes: AQA, BTG, BTR2, BTP, BTY, CCO, TOM, WEN

Data: https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_2421.pdf

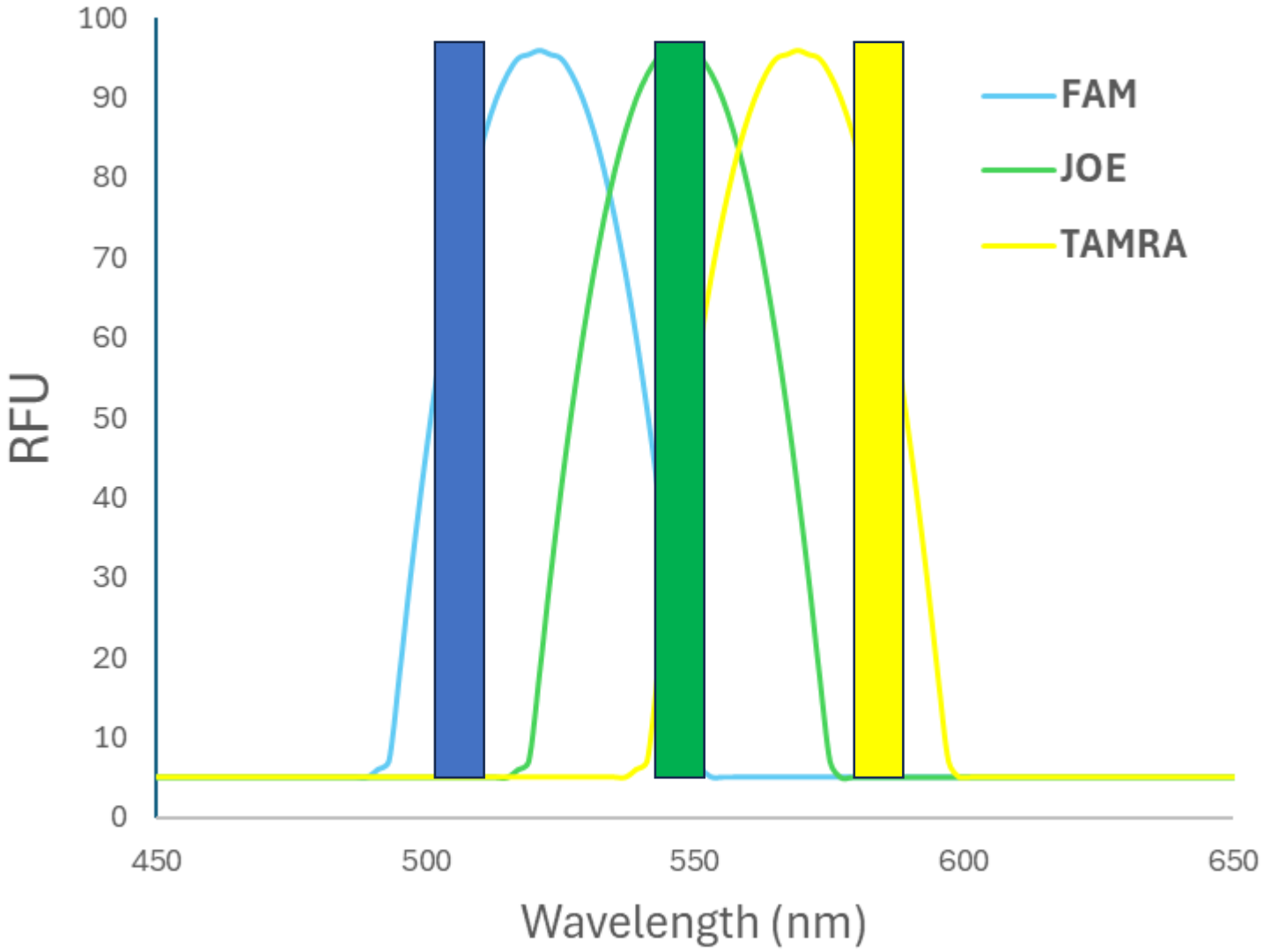
Fluorescent Dye Detection

- Molar absorptivity (ϵ)
  - Measure of how strongly the fluorophore absorbs light
- Quantum Yield (Φ)
  - Ratio of photons emitted/absorbed
  - Measure of the efficiency of converting absorbed photons to light emission
  - Higher quantum yield leads to higher signal intensity
  - Range 0 to 1.0 (100%)
- Highest sensitivity at absorption/emission maxima



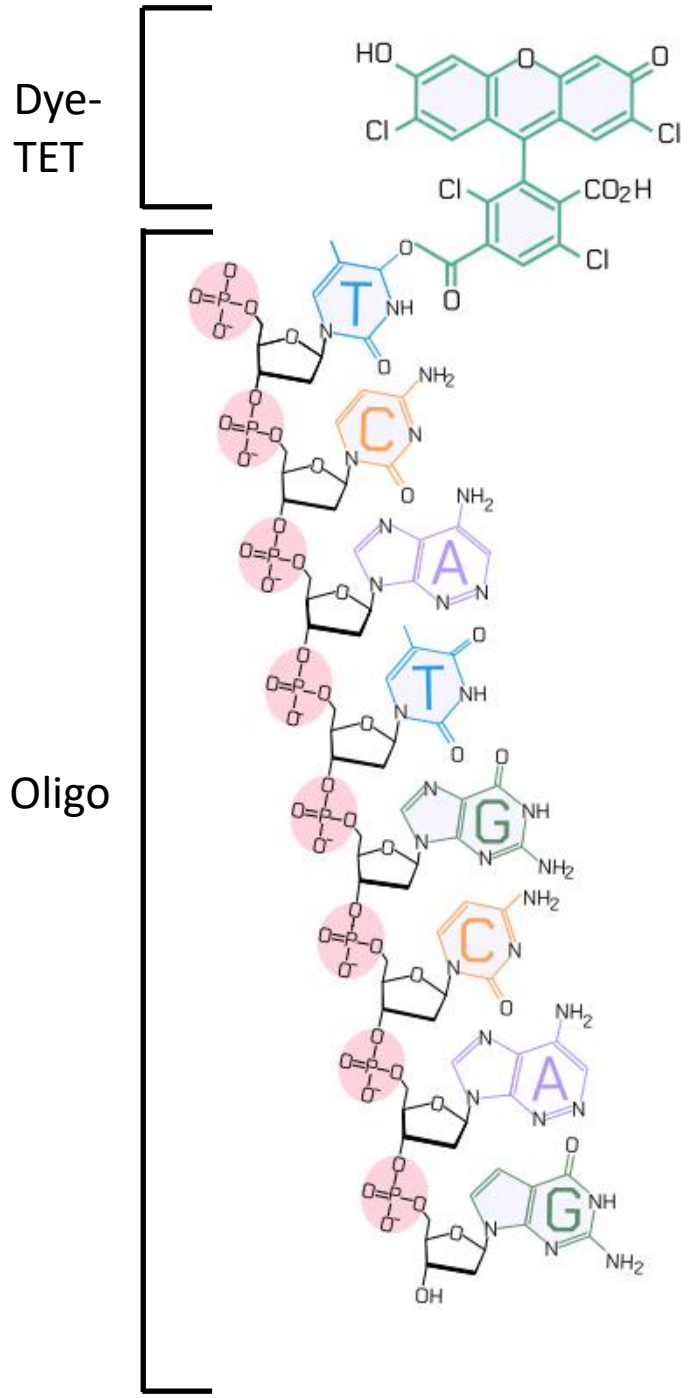
Fluorescent Dye Detection

- Dye overlap
- Excitation
  - 532 nm
- May choose to detect outside of emission maxima
  - Shifting detection wavelength may lead to a loss of sensitivity
- Software deconvolution
  - Matrix



Dye Labelling of PCR Primers

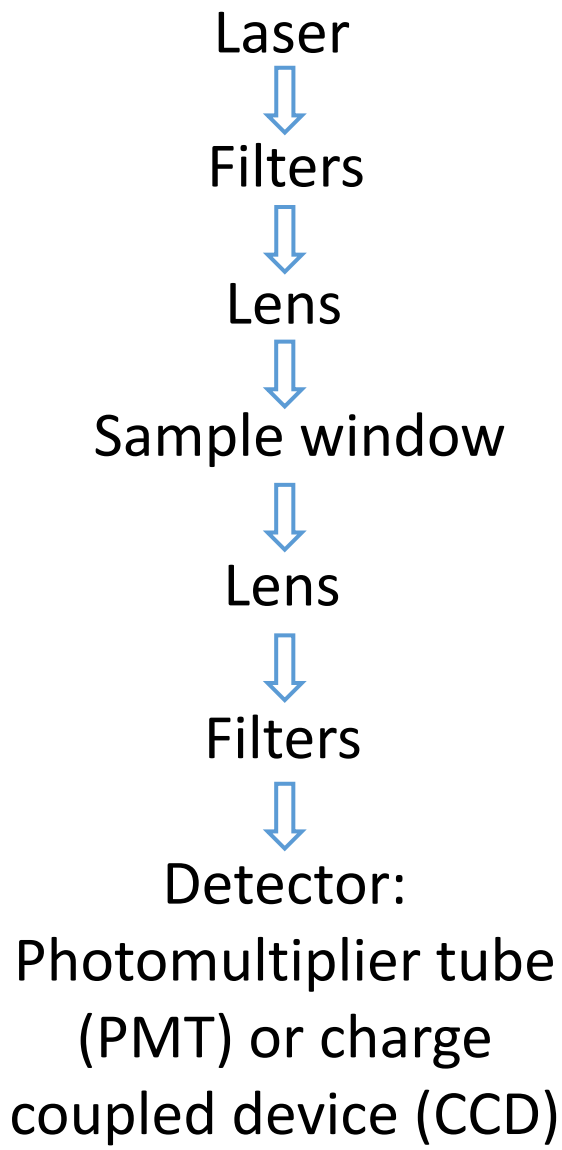
- Dyes can be attached to the 3' and 5' ends of oligos but most frequently the 5' end to enable chain extension at the 3' end
- Modern kits have up to 8 dye detection
- PCR amplification generates a labeled amplicon



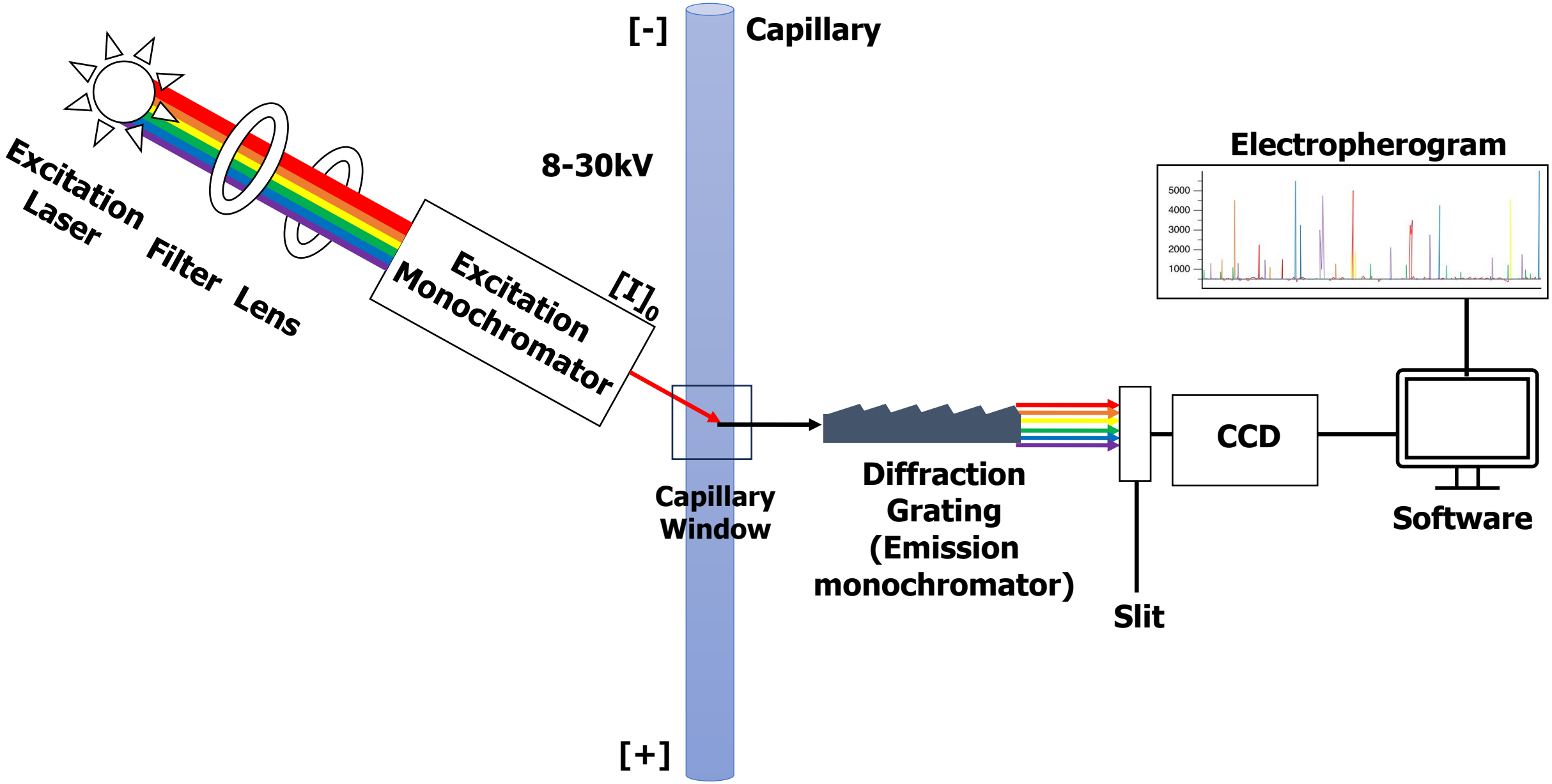
Credit: Nihal Chana

History of STR Typing: Capillary Electrophoresis Detection

- AB 310 CE instrument was introduced in 1995
- Single capillary
- Four dye detection
- Laser induced fluorescence (LIF) detection



CE Detection Schematic



Computer Software Programs for DNA Detection

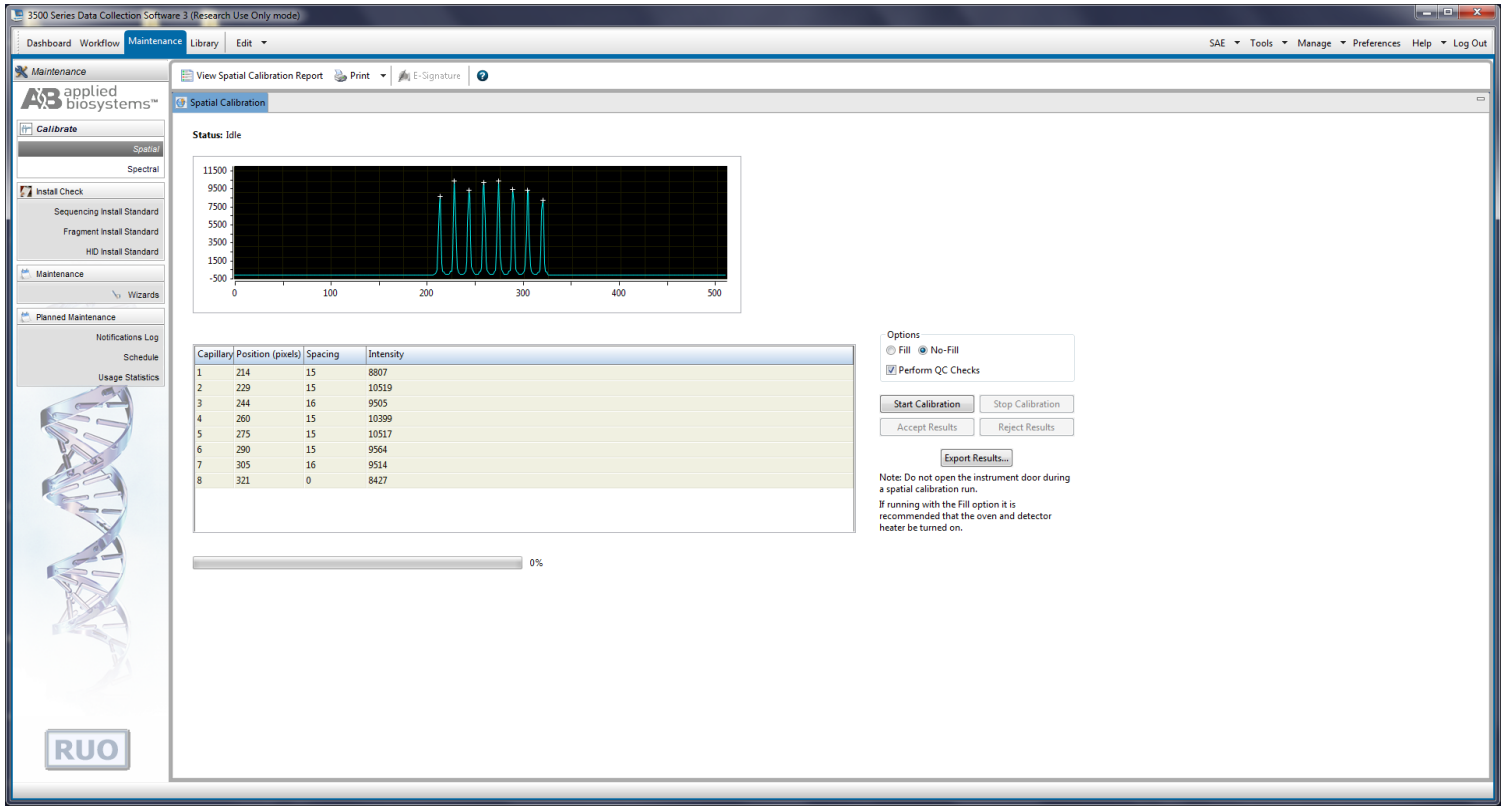
- Applied Biosystems Data Collection and secondary analysis software
  - GeneMapper ID
  - GeneMapper ID-X
- Promega Spectrum Software
- Open Source and Independent Review Interpretation System (OSIRIS)
- TrueAllele by Cybergenetics
- GenoProof 2 and GenoProof Mixture

Number of Dyes Detected in CE Instruments

Capillary Electrophoresis Instrument	Number of Dyes	Number of Capillaries	Manufacturer
310	4	1	Applied Biosystems
3100	5	16	Applied Biosystems
3100-Avant	5	4	Applied Biosystems
3130	5	4	Applied Biosystems
3130xl	5	16	Applied Biosystems
3500	6	8	Applied Biosystems
3500xl	6	24	Applied Biosystems
3730	5	48	Applied Biosystems
3730xl	5	96	Applied Biosystems
SeqStudio	6	4	Applied Biosystems
SeqStudio 8 Flex	6	8	Applied Biosystems
SeqStudio™ 24 Flex	6	24	Applied Biosystems
Spectrum Compact SE	6	4	Promega
Spectrum SE	8	8	Promega

Multicomponent Analysis: Spatial Calibration

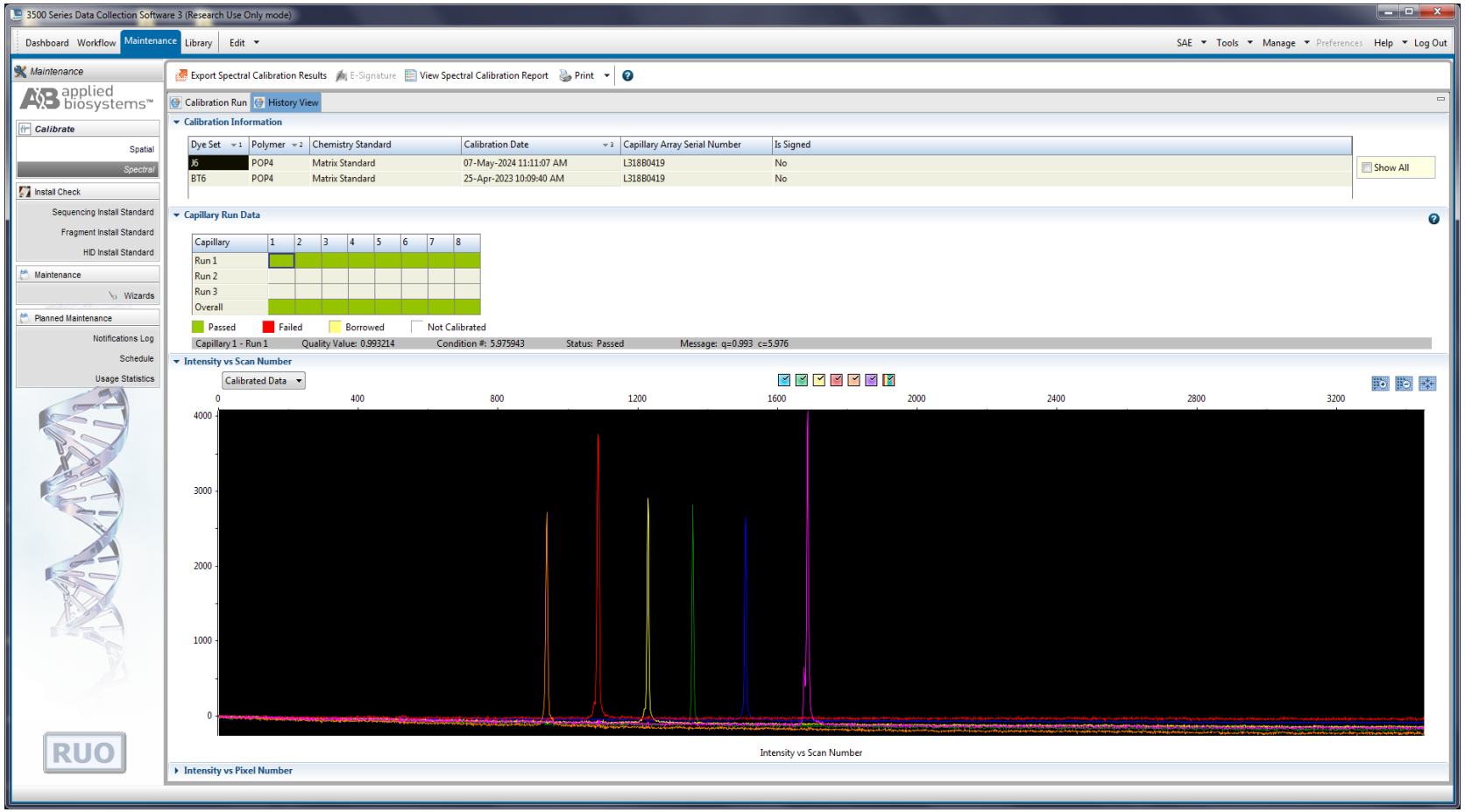
- Physical space
  - Determine relationship between dye elution and signal position detected by the CCD camera



Van Orden A, Keller RA. Fluorescence correlation spectroscopy for rapid multicomponent analysis in a capillary electrophoresis system. Anal Chem. 1998 Nov 1;70(21):4463-71. doi: 10.1021/ac980768q.

Multicomponent Analysis: 6-Dye Spectral Calibration

- Spectral calibration
  - Color deconvolution
  - Injection of dyes into capillary
  - Separate emission files are created for each capillary in an array
  - Must be performed for each dye set



J6 on AB 3500 CE

Spectral Calibration: 8-Dye

Figure 12. Spectral Calibration 'Matrix Data' tab screen.

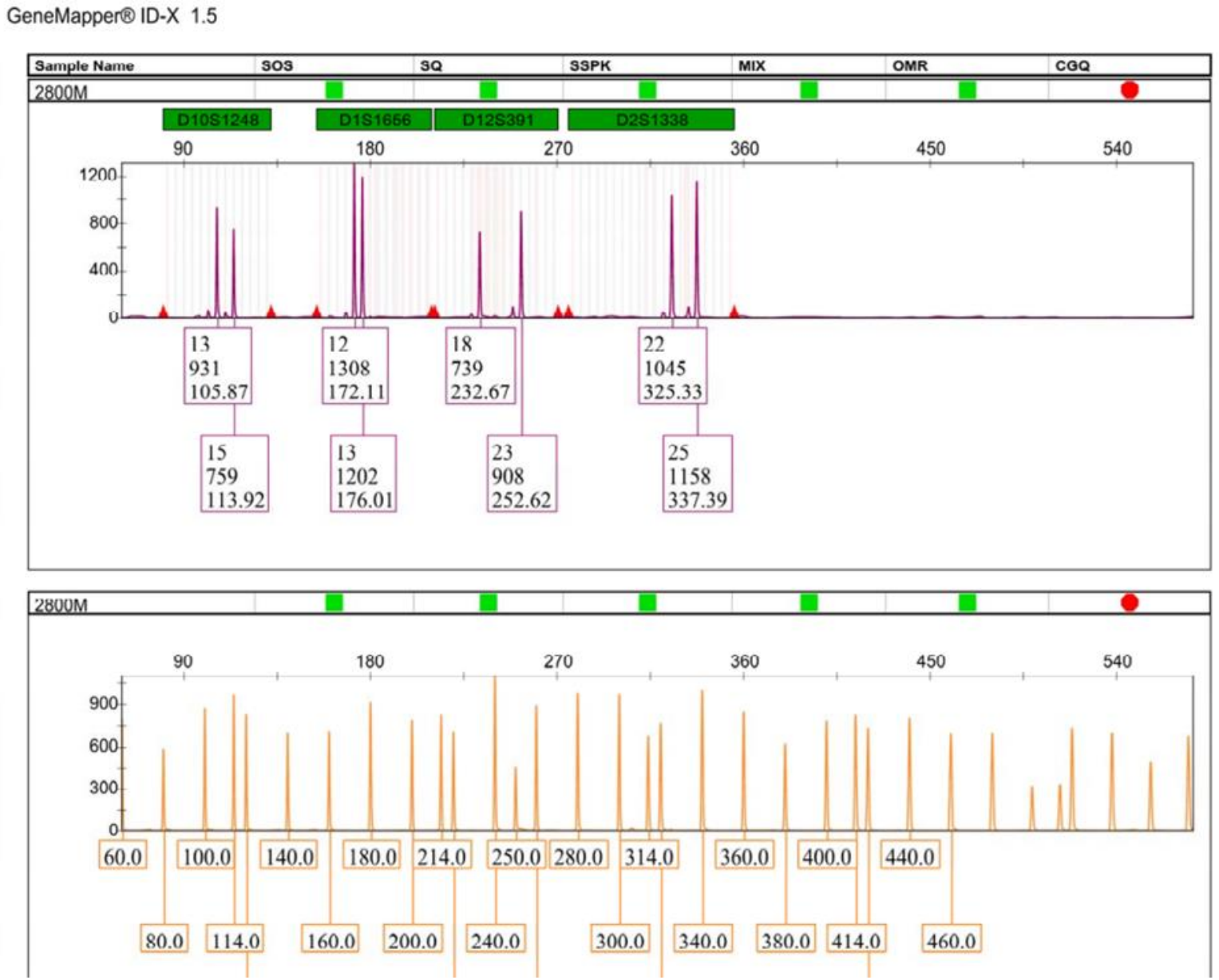
https://www.promega.com/-/media/files/resources/protocols/technical-manuals/tmd/tmd062-powerplex-matrix-standards-for-use-on-the-spectrum-compact-ce-system-technical-manual.pdf?rev=2aba86082a804beaa73fe2d32b252ffb&sc_lang=en

Fluorescence Detection (y-axis)

Allele Sizing using an ISS (x-axis)

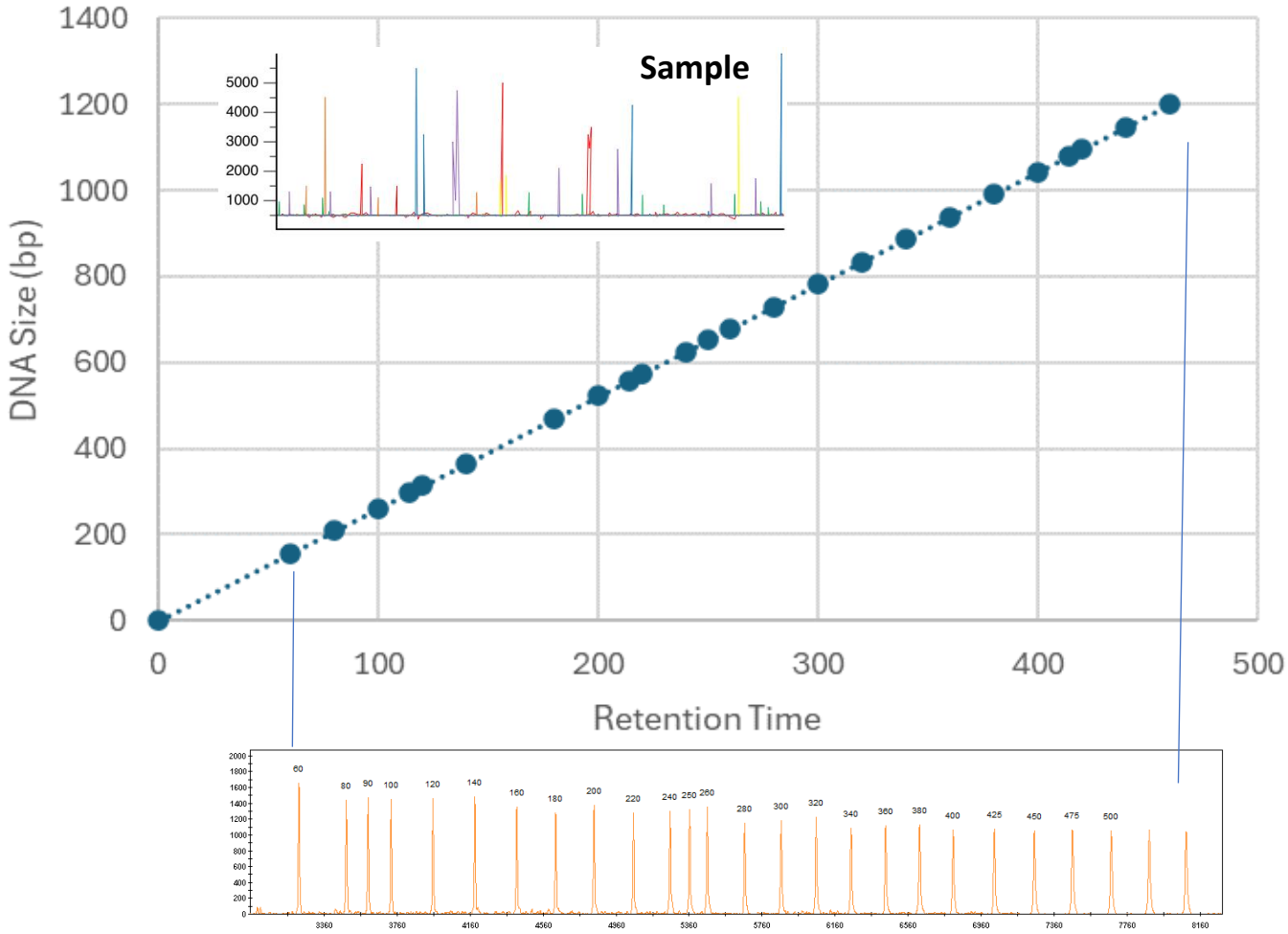
Allele Calling using the Ladder (interpretation)

Analysis of 2800M with GlobalFiler



Fragment Sizing using an Internal Standard

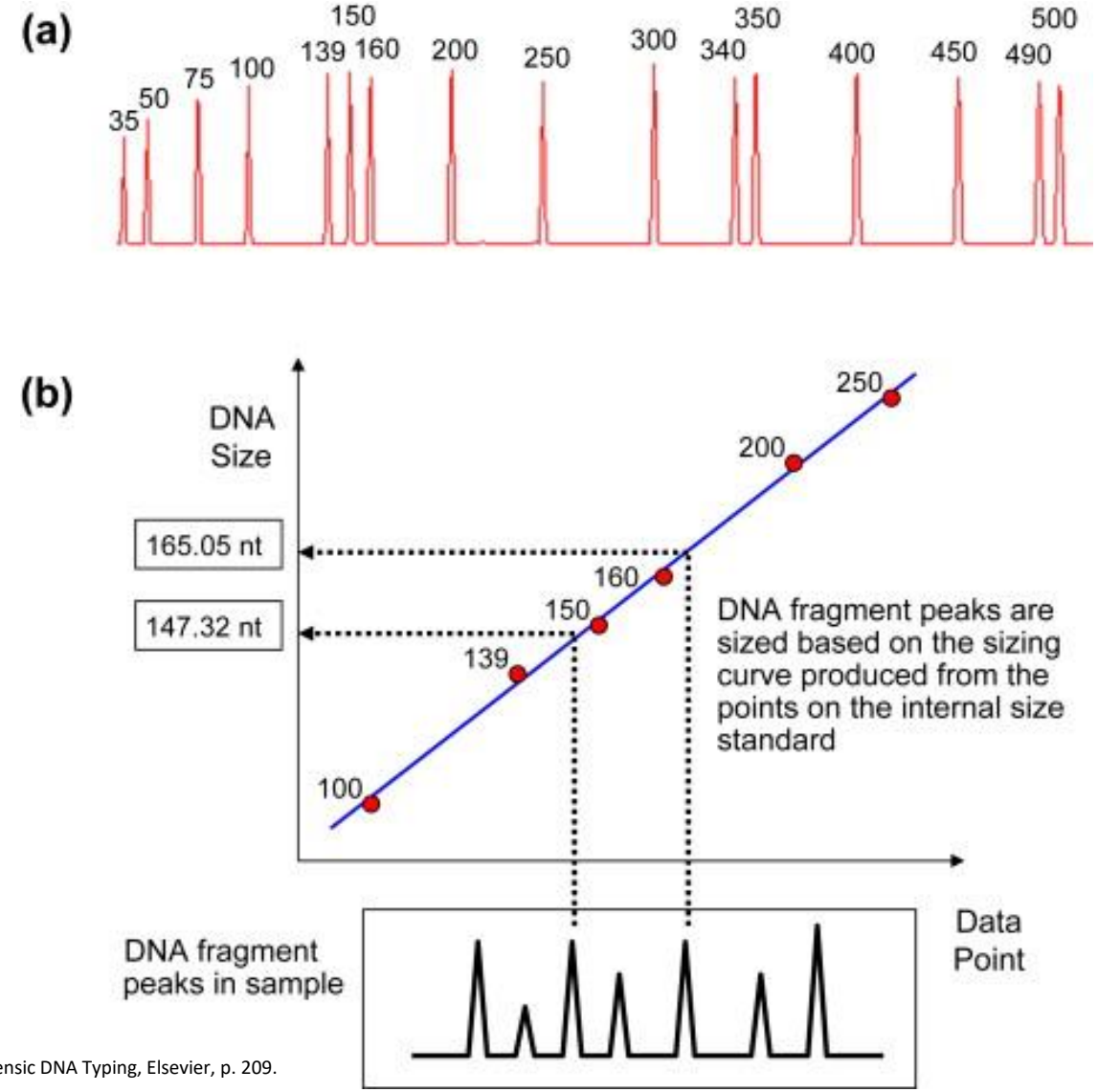
- Internal size standard is added to the sample prior to separation
  - DNA fragments of known size
- Sizing curve is constructed
  - Plot of DNA size vs. migration time for the standard fragments
  - A regression line is constructed and fit is determined



GS600_LIZ 60-460 bp Size Standard

Fragment Sizing using an Internal Standard

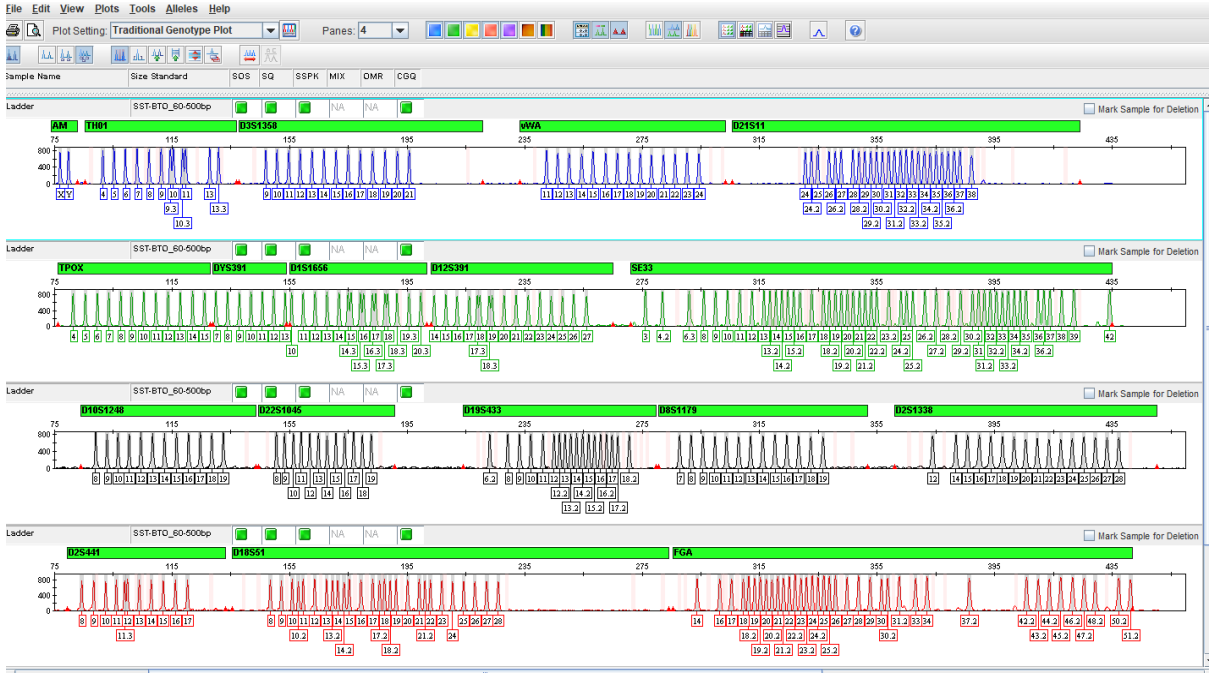
- In the Global Southern Method, the slope is used to compute size of sample fragments using their migration times
- Forensic STR size calculations use Local Southern sizing in which the peaks in the size standard above and below the peak of interest are used to compute the size of the sample peak



Butler, J.M. 2010. Figure 10.3, Fundamentals of Forensic DNA Typing, Elsevier, p. 209.

Allele Calling using a Ladder

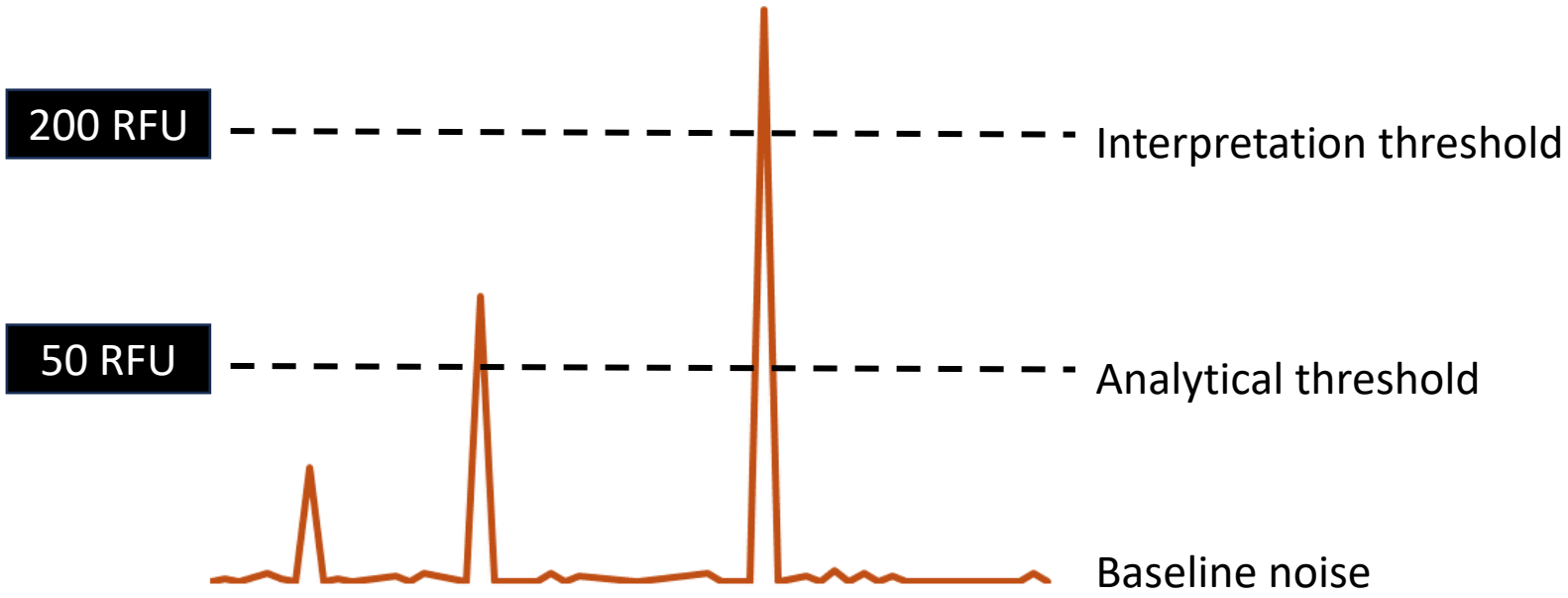
- The allele ladder is a mixture of DNA fragments of known size and sequence.
- It includes known and most common alleles for each locus.
- An internal size standard (bottom panel, orange) is included to correlate the migration time of the ladder peaks to the sample peaks



GlobalFiler Ladder and Internal Size Standard

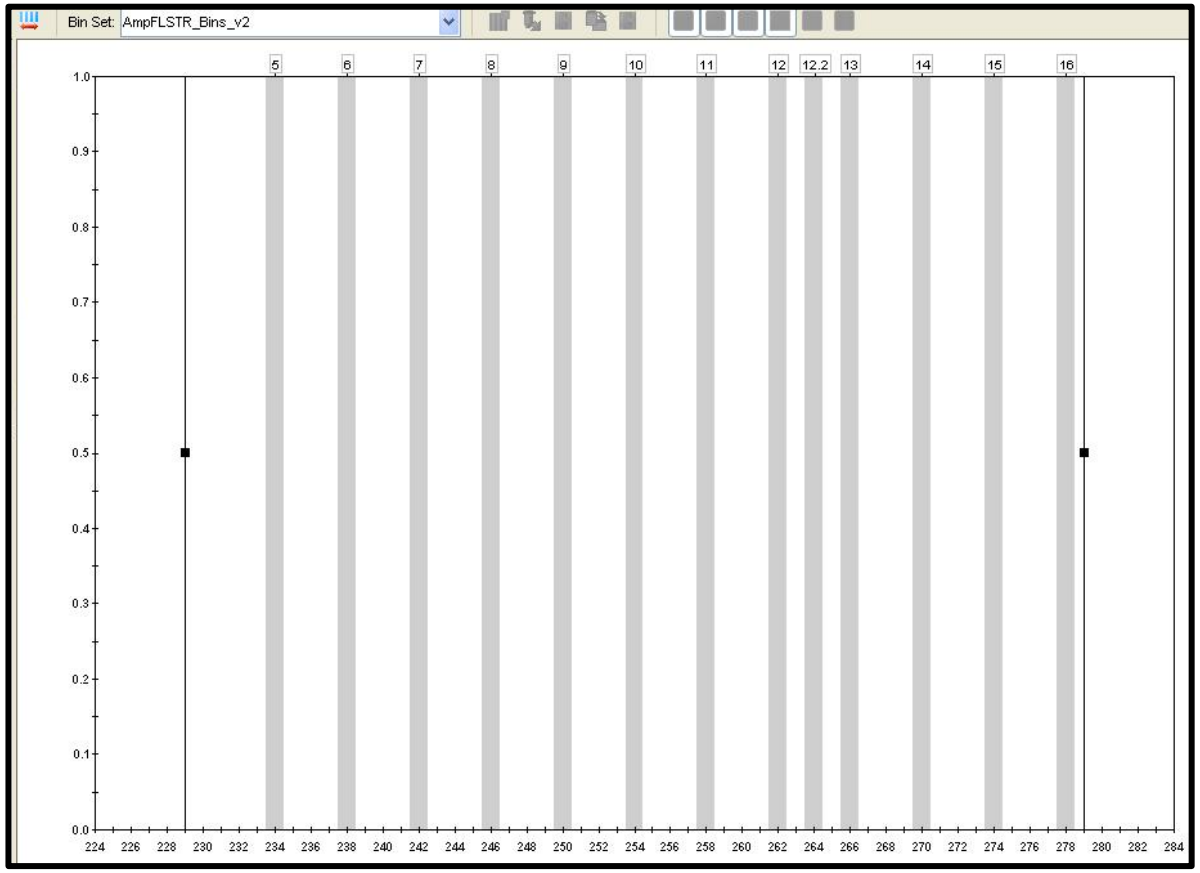
Determining Artifacts from True Alleles

- Peaks above the **interpretation threshold** reproducible and reliable and are called
- The **analytical threshold** is the limit of detection (LOD) and can be used for exclusion
- A peak or baseline noise below the analytical threshold is stochastic and not reliable for interpretation



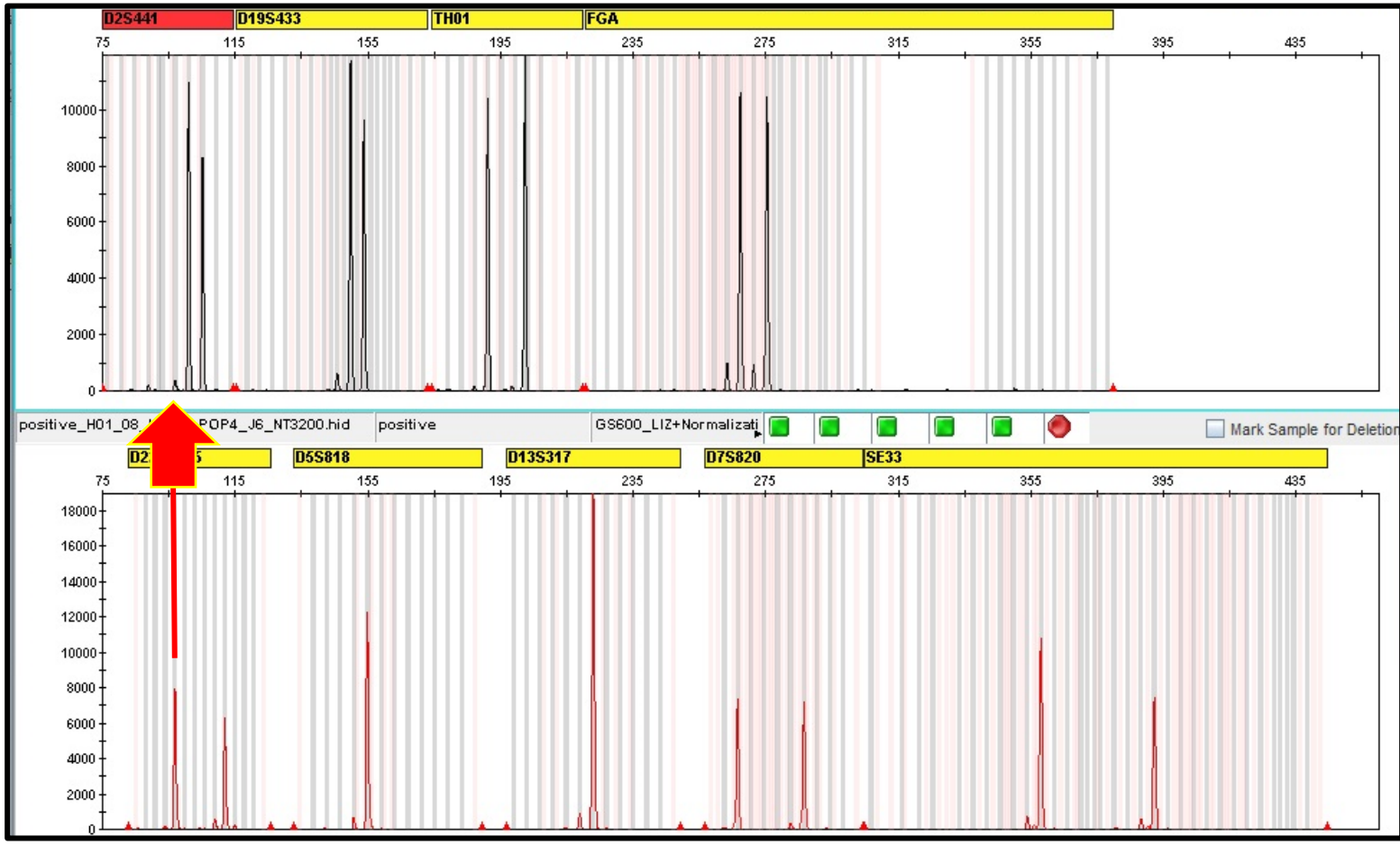
Bins and Virtual Bins

- **Bin** (allele bin)
  - Region that defines an allele within a locus
  - Sensitivity to within 1 bp
  - Separate by 0.1 nt
- **Physical Bin**
  - Region physically defined by the allele ladder
- **Virtual Bin**
  - Alleles not present in the ladder but that have been reported in the literature
- **Bin Offset**
  - Size difference between the physical bin and allele ladder fragments
- **Bin Overlap**
  - Rerun the ladder or replace with one that did not produce overlap



Limitations of the Technology

- Range of visible spectrum wavelengths available limits the range of available colors
- Spectral overlap is not fully removed by matrix deconvolution
- Spectral overlap can lead to pull-up in dye channels
- Size - but not sequence - is determined



Study Questions

- Define fluorescence.
- What methods have been used to detect DNA?
- How can DNA be detected using fluorescence?
- What is a spectral calibration and why is it performed?
- Define analytical threshold.
- Define interpretation threshold.
- How are alleles detected and called?
- Define a bin and virtual bin.
- Using fluorescent STR allele detection technology, how many reaction primers are labeled and what are the dye labels and colors?
- What are limitations of using fluorescence to detect DNA?

Study Questions

- Why are allelic ladders kit specific?
- What can cause a sample not to analyze properly? You are troubleshooting a CE run. What are possible explanations for the following observations?
  - No PCR product is present in all lanes, but the size standards are visible.
  - Most samples look OK, but one sample has neither standard nor alleles.
  - The smaller fragments peaks are sharp but later peaks get progressively lower and wider.
  - All of the peaks are present in all of the color channels.
  - There are spikes in almost every lane.

Suggested Readings

- ANSI/ASB Standard 115, Standard for Training in Forensic Short Tandem Repeat Typing Methods using Amplification, DNA Separation, and Allele Detection. 2020. 1st Ed. https://www.aafs.org/sites/default/files/media/documents/115_Std_e1.pdf
- Butler, J.M. Advanced Topics in Forensic DNA Typing: Methodology, Elsevier, 2011.
- FBI, Quality Assurance Standards for Forensic DNA Testing Laboratories (QAS), effective September 1, 2011.